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Keywords: light regulation; marine cyanobacteria; photosynthesis; phycobilin lyase; phycoerythrin

Abbreviations : BL, blue light; CA4, Type IV chromatic acclimation; EICs, extracted ion chromatograms; GL, green light; HT-, hexahistidine-tagged; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MW, molecular weight; PBS, phycobilisome; PC, phycocyanin; PCB, phycocyanobilin; PEB, phycoerythrobilin; PEI, phycoerythrin-I; PEII, phycoerythrin-II; PT, pigment type; PUB, phycourobilin

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Abstract

Marine *Synechococcus* cyanobacteria owe their ubiquity in part to the wide pigment diversity of their light-harvesting complexes. In open ocean waters, cells predominantly possess sophisticated antennae with rods composed of phycocyanin and two types of phycoerythrins (PEI and PEII). Some strains are specialized for harvesting either green or blue light, while others can dynamically modify their light absorption spectrum to match the dominant ambient color. This process called 'Type-IV chromatic acclimation' (CA4) has been linked to the presence of a small genomic island occurring in two configurations (CA4-A and -B). While the CA4-A process has been partially characterized, the CA4-B process has remained an enigma. Here, we characterize the function of two members of the phycobilin lyase E/F clan, MpeW and MpeQ, in *Synechococcus* sp. strain A15-62 and demonstrate their critical role in CA4-B. While MpeW, encoded in the CA4-B island and upregulated in green light, attaches the green-light absorbing chromophore phycoerythrobilin to cysteine-83 of the PEII α-subunit in green light, MpeQ binds phycoerythrobilin and isomerizes it into the blue-light absorbing phycourobilin at the same site in blue light, reversing the relationship of MpeZ and MpeY in the CA4-A strain RS9916. Our data thus reveal key molecular differences between the two types of chromatic acclimaters, both highly abundant but occupying distinct complementary ecological niches in the ocean. They also support an evolutionary scenario whereby CA4-B island acquisition allowed former blue-light specialists to become chromatic acclimaters, while former green-light specialists would have acquired this capacity by gaining a CA4-A island.

Significance

Of all cyanobacteria on Earth, marine *Synechococcus* are those displaying the greatest pigment diversity. The most sophisticated pigment type are cells able to reversibly modify their color by a phenomenon called Type

IV chromatic acclimation or CA4. Two genetically distinct CA4 types (CA4-A and CA4-B) have evolved in different lineages. Together, they represent almost half of all *Synechococcus* cells in oceanic areas, and are equally abundant but occupy complementary ecological niches. While the molecular mechanism of CA4-A has recently started to be deciphered, the CA4-B mechanism was so far uncharacterized. Here, by unveiling this mechanism and demonstrating its singularity relative to CA4-A, we provide novel highlights on the evolutionary history of *Synechococcus* acclimation to light color in the oceans.

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Introduction

Marine *Synechococcus* are the second most abundant phototrophs in the oceans, significantly contributing to oceanic primary production and carbon cycling (1, 2). These picocyanobacteria occupy the upper lit layer of marine waters from the equator up to 80 °N (3, 4) and exhibit a broad range of photosynthetic pigments, allowing them to optimally exploit a large variety of light environments, from particle-loaded estuaries to the optically clear open ocean waters (5). This pigment diversity arises from a large flexibility in the composition of their light-harvesting antennae, called phycobilisomes (PBS). Structurally, these complexes comprise a central core from which radiate six or eight rods made of α/β heterodimers of phycobiliproteins that first assemble into $(\alpha\beta)_6$ hexamers and then stack into rods with the assistance of linker proteins (6). In the open ocean, virtually all *Synechococcus* cells belong to pigment type 3 (PT 3), in which PBS rods are made of the three phycobiliproteins, phycocyanin (PC), phycoerythrin-I (PEI) and phycoerythrin-II (PEII), which can covalently bind three chromophore types: the red-light absorbing phycocyanobilin (PCB, A_{max} = 650nm), the green-light (GL) absorbing phycoerythrobilin (PEB, A_{max} = 550 nm) and the blue-light (BL) absorbing phycourobilin [PUB, A_{max} = 495 nm (7, 8)]. Different pigment subtypes have been defined within PT 3, based

on the relative ratio of fluorescence excitation at 495 nm and 545 nm with emission at 580 nm ($Exc_{495:545}$), a proxy for their molar PUB:PEB ratio. This ratio is constitutively low ($Exc_{495:545} < 0.6$), intermediate ($0.6 \leq Exc_{495:545} < 1.6$) or high ($Exc_{495:545} \geq 1.6$) in PT 3a, 3b and 3c, respectively (5, 9). Furthermore, in PT 3d strains, which appear to make up nearly half of the PT 3 population globally (9), the PUB:PEB ratio can reversibly vary with ambient light color, a process called type IV chromatic acclimation (CA4) that allows cells to optimally absorb either GL or BL, depending on the ambient ratio of these light colors (10, 11). While most genes coding for PBS rod components are localized in a large genomic region, called the PBS region (5), strains capable of CA4 additionally possess a small genomic island existing in one of two distinct configurations (12). Each contains the two regulators encoded by *fciA* and *fciB* (13) and one gene coding for a putative phycobilin lyase family member, which can be either *mpeZ* or *mpeW*, defining the two distinct genotypes CA4-A and CA4-B [the corresponding PTs are named 3dA and 3dB, respectively; (12)]. Phycobilin lyases are enzymes responsible for the covalent attachment of chromophores at specific cysteine residues of phycobiliprotein subunits and are thus key players in *Synechococcus* pigmentation. Indeed, chromophore ligation takes place prior to phycobiliprotein subunit assembly, and proper phycobilin arrangement is crucial for efficient energy transfer along the rods (14). However, among the dozen (putative) phycobilin lyases encoded in marine *Synechococcus* genomes (5), only a few have been biochemically characterized. RpcG from *Synechococcus* sp. WH8102 (PT 3c) was shown to attach PEB to cysteine-84 (C84) of the α-subunit of PC and to isomerize it to PUB (15). Such dual-function enzymes are called phycobilin lyase-isomerases. More recently, the biochemical characterization of the CA4-A strain *Synechococcus* sp. RS9916 (clade IX) revealed the specific chromophore changes that occur during CA4, namely one out of five chromophore-binding sites on PEI and two of the six sites of PEII change from PEB in GL to PUB in BL. Two enzymes implicated in CA4-A and belonging to the E/F structural clan (16) have been characterized: MpeZ is the lyase-isomerase adding PUB at C83 of the PEII α-subunit (MpeA) in BL (17), whereas its paralog MpeY is the PEB lyase acting on the same site in GL (18). While *mpeZ* was found to be expressed more highly under BL (12, 13, 17), *mpeY* is

constitutively expressed whatever the ambient light color. This led to the suggestion that the cellular MpeZ:MpeY protein ratio ultimately controls the chromophore bound at MpeA-C83 (18). By contrast, the identity and function of the corresponding enzymes in CA4-B strains was so far unknown.

Here, we characterize the *mpeW* gene located in the CA4 genomic island of the CA4-B strain *Synechococcus* sp. A15-62 (sub-clade IIc). We show that it encodes a phycobilin lyase acting on MpeA-C83 in GL that vies a previously unidentified lyase-isomerase (MpeQ) acting on the same cysteine position in BL. Our results highlight the key role of the diversification of the MpeY enzyme family in the adaptation of marine *Synechococcus* to different light color niches and provide new insights to explain the occurrence of two mutually exclusive CA4 systems in marine *Synechococcus*.

Results

The MpeY Phycobilin Lyase Family Is Polyphyletic. Based on its conserved genomic position in the PEII-specific sub-region of the PBS region (Fig. 1A) and on sequence similarity, the gene located immediately upstream of the *mpeBA* operon (encoding the two subunits of PEII) was previously named *mpeY* in all *Synechococcus* strains containing PEII (5, 9, 18). However, the phylogenetic analysis of the different members of this family revealed that *mpeY* is in fact polyphyletic, with three distinct clusters, each corresponding to a different pigment subtype (Fig. 1C). PT 3a strains (i.e., GL specialists) possess the MpeY^{3a} variant encoded by the PT 3a-specific *mpeY* allele, PT 3dA strains contain the MpeY^{3dA} variant recently characterized in the PT 3dA strain *Synechococcus* sp. RS9916 as a PEB lyase acting on C83 of the α-PEII subunit (MpeA) (18), while a third variant is found in PT 3c (i.e., BL specialists) and 3dB strains. Strikingly, sequences from the latter two pigment sub-types were intermingled in the phylogenetic tree, as was previously observed for *mpeBA* or *cpeBA* phylogenies, indicating that PBS genes of these pigment subtypes are phylogenetically too close to be distinguishable (9, 12). Tandem mass spectrometry analysis of the chromophorylation of MpeA from the PT

3a strain *Synechococcus* sp. WH7803 revealed that MpeA-C83 binds a PEB, while PUB is bound to both MpeA-C75 and 140 (*SI Appendix,* Fig. S1). This suggests that MpeY^{3a} must have a PEB lyase function, like MpeY^{3dA}. In contrast, as demonstrated below, the function of the MpeY-like protein found in PT 3c and 3dB differs from that of both MpeY^{3a} and MpeY^{3dA} and we therefore renamed it 'MpeQ' in the tree (Fig. 1C) and in the text hereafter. The protein sequences of MpeW, MpeZ, MpeQ, MpeY^{3a} and MpeY^{3dA} have on average 34-58% amino acid identity over their whole length, compared to an average of 70-82% identity amongst members of each of these sub-families (Fig. 1B and Dataset S1).

Chromophore Changes Occurring During CA4-B Are the Same as Those Occurring in the CA4-A Process. Although the $Exc_{495:545}$ ratios of CA4-A and -B strains have previously been shown to be similar in both BL (1.6-1.7) and GL (0.6-0.7) (12), it was unclear what specific changes in chromophorylation were taking place during the CA4 process in CA4-B strains. To investigate this, PBS from wild-type *Synechococcus* sp. A15-62 grown under BL or GL were purified, and PEI and PEII subunits separated by HPLC. Absorption spectra of individual subunits (Fig. 2 and *SI Appendix,* Fig. S2) suggested that the PUB:PEB molar ratio changed between GL and BL from 0:2 to 1:1 for the α-PEI subunit (CpeA) and from 1:2 to 3:0 for the α-PEII subunit (MpeA), while the chromophorylation of β-PEI (CpeB) and β-PEII (MpeB) subunits did not vary with light color (1:2 in both conditions; Table 1). The chromophorylation of the two α-subunits was further characterized by tandem mass spectrometry, confirming the abovementioned molar ratios and demonstrating that the three cysteine positions that change chromophorylation (from PEB in GL to PUB in BL) are MpeA-C83 and C140 and CpeA-C139 (Table 1, Dataset S2 and *SI Appendix,* Figs. S3-S5), i.e. the same chromophores and positions as in the CA4-A strain RS9916 (17).

MpeW and MpeQ Are Required for PEB and PUB Attachment on MpeA, Respectively. Since the biochemical characterization of MpeZ and MpeY^{3dA} in RS9916 showed that they were respectively a PEB lyase-isomerase and a PEB lyase, both acting on MpeA-C83 (17, 18), we hypothesized that the corresponding enzymes in CA4-B strains, MpeW and MpeQ (Fig. 1A), could be a PEB lyase and PEB lyase-isomerase, respectively, both also acting on MpeA. To test this hypothesis, we created *mpeW*$^-$ and *mpeQ*$^-$ insertion mutants in the CA4-B strain A15-62. Comparison of fluorescence excitation spectra revealed that for GL-grown cultures, $Exc_{495:545}$ was increased in the *mpeW*$^-$ mutant compared to the wild-type (WT), whereas no difference was observed under BL (Fig. 3A-B). Conversely, no difference could be seen between $Exc_{495:545}$ of *mpeQ*$^-$ mutants and WT under GL, while the $Exc_{495:545}$ ratio dramatically decreased in BL-grown *mpeQ*$^-$ mutants compared to WT (Fig. 3C-D). Both mutants still exhibited some levels of chromatic acclimation, since the $Exc_{495:545}$ ratio changed from 1.17 ± 0.01 in GL to 1.68 ± 0.07 in BL for *mpeW*$^-$ and from 0.74 ± 0.02 in GL to 0.91 ± 0.01 in BL for *mpeQ*$^-$, as compared to 0.74 ± 0.01 in GL and 1.72 ± 0.09 in BL for WT cells (all data are average ± SE; n = 2). Based on these results, we hypothesized that i) MpeW is required for PEB attachment, while MpeQ is required for PEB attachment and isomerization to PUB and ii) both are acting on one or possibly two chromophore binding cysteine(s), but seemingly not all three sites that change chromophorylation during the CA4 process. The CA4 phenotype of both mutants could be restored with the re-introduction of WT *mpeW* or *mpeQ* genes in *trans* (Fig. 3 and *SI Appendix,* Fig. S6).

Since in the CA4-A strain RS9916, the transcript levels of the lyase-isomerase *mpeZ* were found to be about 32-fold higher in BL than GL (12), while the *mpeY* lyase levels were similar in both colors (18), we also checked the light color response of the counterparts *mpeQ* and *mpeW* genes in the CA4-B strain A15-62. Real-time PCR expression data showed that while *mpeQ* is not significantly differentially regulated between GL and BL, *mpeW* is 44.26 ± 1.34-fold (average ± SD; n=3) more expressed in GL than BL (*SI Appendix,* Fig. S7).

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158 ***mpeW*$^{-}$ and *mpeQ*$^{-}$ Mutants Differ from the WT by the Chromophore Attached to MpeA-C83 in Green and**
159 **Blue Light, Respectively.** To identify the biochemical changes responsible for the differences in whole-cell
160 fluorescence, PBS were purified from the *mpeW*$^{-}$ and *mpeQ*$^{-}$ mutants and PEI and PEII subunits separated by
161 HPLC. Absorption spectra only differed between WT and mutants for MpeA (Fig. 2 and *SI Appendix,* Fig. S2).
162 Indeed, compared to the WT, MpeA from the *mpeW*$^{-}$ mutant had a lower PEB absorption peak at 550 nm in
163 GL (Fig. 2A), whereas MpeA from the *mpeQ*$^{-}$ mutant exhibited a PEB peak at 550 nm in BL not seen in WT
164 cells (Fig. 2D). This confirmed that both MpeW and MpeQ are involved in MpeA chromophorylation, with
165 MpeW being responsible for PEB attachment and MpeQ for PEB attachment and simultaneous isomerization
166 to PUB. Tandem mass spectrometry of CpeA and MpeA, the two PE subunits that change chromophorylation
167 during CA4 (see above), further showed that the *mpeW*$^{-}$ mutant mainly differed from the WT by having a
168 PUB instead of a PEB at MpeA-C83 in GL, whereas the *mpeQ*$^{-}$ mutant differed from the WT by having a PEB
169 instead of a PUB at MpeA-C83 in BL (Table 1 and *SI Appendix*, Fig. S8-9). Surprisingly, both WT and mutants
170 displayed a mixed chromophorylation at MpeA-C140 in GL. The slightly higher proportion of PUB in mutants
171 compared to the WT (35% and 15%, respectively; Table 1) could suggest that MpeQ and MpeW might also
172 have a role in the chromophorylation changes occurring during CA4 at this position. If true, this effect is likely
173 indirect, since no chromophorylation of MpeA-C140 could be detected in our recombinant assays (see
174 below).

175

176 **Recombinant MpeW and MpeQ are Both Acting on MpeA-C83.** Activities of MpeW and MpeQ were tested
177 using a heterologous plasmid coexpression system in *Escherichia coli*. Four different hexahistidine-tagged
178 (HT-) versions of recombinant MpeA were used as substrates in the coexpressions: A15-62 and RS9916 HT-
179 MpeA; a mutant RS9916 MpeA with C83 replaced by alanine, RS9916 HT-MpeA(C83A); and a mutant RS9916

MpeA with C75 and C140 each replaced with alanine, RS9916 HT-MpeA(C75A, C140A). RS9916 HT-MpeB and RS9916 HT-CpeA were also used to further test the substrate preference. These phycobiliprotein subunits were co-expressed with PEB synthesis enzymes and with either MpeW or MpeQ, purified and their spectral properties analyzed. The negative control (A15-62 HT-MpeA expressed with PEB synthesis enzymes but without any lyase) showed no detectable fluorescence (Fig. 4A, C). The coexpressions of MpeA (either from A15-62 or RS9916) and either MpeW or MpeQ confirmed that the former attached PEB while the latter attached PUB to both versions of MpeA (Fig. 4A-D), while coexpressions of MpeW or MpeQ and either MpeB or CpeA demonstrated that neither had lyase activity on these two proteins. Coexpressions of MpeW and mutants RS9916 MpeA, in which cysteine residues are replaced by alanine [RS9916 MpeA (C83A) or (C75A, C140A)] further showed that PEB was covalently bound to MpeA-C83 (Fig. 4A-B). The same system was used to demonstrate that MpeQ also acted specifically on MpeA-C83 by ligating PEB and isomerizing it to PUB (Fig. 4C-D). The minor fluorescence emission peak at ~600 nm in the *mpeW* system (Fig. 4A) is likely coming from PEB that is not held in a stretched conformation.

Collectively, these results indicate that in the CA4-B process, MpeW is the lyase responsible for the attachment of PEB to MpeA-C83 in GL while MpeQ is the lyase-isomerase responsible for the attachment of PEB and its isomerization to PUB at MpeA-C83 in BL.

Discussion

With six phycobilin binding sites per $\alpha\beta$ monomer, PEII is the most pigmented phycobiliprotein known so far in the *Cyanobacteria* phylum (7, 19), and the ecological success of marine *Synechococcus* spp. is likely tied

with this biological innovation, as evidenced by the large predominance of PEII-containing cells (so-called PT 3) in open waters of the world ocean (9). Gain of PEII, which is thought to have occurred by duplication and divergence from the corresponding PEI encoding genes (20), also implied the concomitant acquisition of enzymes necessary for its proper chromophorylation (5). In this context, the diversification of the MpeQWYZ enzyme family, which belongs to the E/F structural clan (16) and likely derived by duplication and divergence from a *cpeY*- like gene ancestor (Fig. 1) (21, 22), has been pivotal for the development of CA4, the only example among the six chromatic acclimation forms known so far in *Cyanobacteria* to involve color-induced changes only in chromophore content (23). The fact that CA4 seemingly appeared twice during the evolution of marine *Synechococcus*, with CA4-A and -B occurring in most cases in phylogenetically distant lineages and colonizing different light niches (Fig. 1) (9, 12) is particularly intriguing. Although the molecular mechanism underpinning CA4-A is starting to be understood (13, 17, 18), CA4-B was thus far biochemically uncharacterized, and the differences between the two CA4 systems remained unclear. Here, we show that CA4-B is mechanistically similar to CA4-A, since it also involves two enzymes that vie for the same binding site on α-PEII subunit (MpeA) and whose gene expressions are differentially regulated by the ambient light color (*SI Appendix*, Fig. S10). We demonstrate that MpeW is a phycobilin lyase attaching PEB at MpeA-C83 in GL, whereas MpeQ is a phycobilin lyase-isomerase binding PUB at the same position in BL. Intriguingly, the CA4‒B-specific lyase/lyase-isomerase MpeW/Q pair exhibits a reversed activity compared to its counterpart pair MpeZ/Y in the CA4-A strain RS9916 (*SI Appendix*, Fig. S10). Indeed, the lyase-isomerase *mpeZ* was found to be upregulated under BL, while in the CA4-B process the lyase *mpeW* is upregulated under GL. In both cases, expression of the gene coding for the second enzyme of the couple is not differentially expressed between GL and BL (12, 13, 17). Our data therefore sheds light on a fascinating dual-enzyme co-evolution process that explains the co-occurrence of two mutually exclusive CA4 types in the marine *Synechococcus* radiation. Our data provide strong evidence for the previously proposed hypothesis (23, 24) that the “basal state” for CA4-A strains (i.e., in absence of CA4) corresponds to a GL specialist phenotype (low PUB:PEB ratio)

and that CA4 confers these cells the ability to change their spectral properties upon BL illumination, by inducing the expression of the phycobilin lyase-isomerase MpeZ. Conversely, the "basal state" for CA4-B strains would correspond to a BL specialist phenotype (high PUB:PEB ratio), CA4 providing these cells the ability to acclimate to GL by inducing the synthesis of the PEB lyase MpeW. Thus, in both cases, CA4 appears to be a "plug-and-play" mechanism that adds on top of the existing basal chromophorylation. This view is further supported by the gathering into a small dedicated genomic island of genes necessary for CA4, including the regulators *fciA* and *fciB* and one phycobilin lyase (either *mpeZ* or *mpeW*; Fig. 1A). Furthermore, the absence of any allelic difference between PBS genes of PT 3c and 3dB strains (Fig. 1C; see also (9)) might suggest that the CA4-B island is more readily transferred between *Synechococcus* strains through horizontal gene transfer than is the CA4-A island. Surprisingly however, the latter displays several typical genomic island features, including a biased GC content compared to the surrounding genomic context and presence in the vicinity of the island of hotspots for DNA recombination such as tRNA or *psbA* genes, which are absent in the case of the CA4-B island (12, 17). So, the mechanism by which a CA4-B island could be transferred from a PT 3dB strain to a BL specialist (PT 3c) and the frequency of such transfers remain unclear.

It is also interesting to note that, as previously observed for *mpeZ*$^{-}$ and *mpeY*$^{3dA-}$ mutants in the CA4-A strain RS9916 (17, 18), both *mpeW*$^{-}$ and *mpeQ*$^{-}$ CA4-B mutants still showed some degree of chromatic acclimation, variations of the $Exc_{495:545}$ ratio observed in the *mpeW*$^{-}$ and *mpeQ*$^{-}$ mutants representing about 51% and 18% of that occurring in the WT, respectively (Fig. 2C-D). In both lyase-isomerase mutants (*mpeZ*$^{-}$ and *mpeQ*$^{-}$), $Exc_{495:545}$ is strongly decreased in BL (about 0.8) compared to the WT (1.5-1.6), but higher than in GL (0.6-0.7 for both mutants and WT; Fig. 2C-D and (17)). Similarly, both lyase mutants (*mpeY*$^{-}$ and *mpeW*$^{-}$) had an increased $Exc_{495:545}$ in GL relative to the WT (about 1.1 vs. 0.6), but still lower than in BL [1.5-1.6 for both; Fig. 2A-B and (18)]. This suggests that other genes are involved in CA4. In the absence of any obvious other CA4-specific phycobilin lyase, an interesting candidate could be the conserved hypothetical gene *unk10*, which is present in both versions of the CA4 genomic island and strongly overexpressed in BL in the

CA4-A strain RS9916 (12, 17, 23). Future characterization of this unknown gene as well as the putative regulator *fciC*, present in CA4-A but not CA4-B islands (Fig. 1A and *SI appendix* Fig. S10), will help unravel the different regulatory mechanisms behind this remarkable dual light color acclimation process, which strongly impact the ecology and photophysiology of a key component of marine phytoplankton communities.

Materials and Methods

Phylogenetic Analyses. Sequences used in this study (MpeQ, W, Y, Z) can be found in Dataset S1. Briefly, MpeQ, W, Y and Z protein sequences were aligned using MAFFT 7.299b L-INS-I algorithm (25). Pairwise identity between sequences was computed from this alignment using BioPython. Best model for phylogenetic reconstruction corresponded to LG aa substitution model with empirical base frequencies (F), gamma model of rate heterogeneity (G) and invariable sites (I) as selected with ProtTest 3.4.1 (26). Tree search was conducted using RAxML 8.2.9 (27) without the rapid hill-climbing heuristic (-f o). 100 tree searches were conducted starting from randomized maximum parsimony tree (default), and 100 searches starting from fully random trees (-d). Best tree was selected and 250 bootstraps performed. Tree was plotted using ETE3 (28). The same procedure was used to generate a tree with CpeY sequences to root the tree represented in Fig. 1C.

Strains and Growth Conditions. *Synechococcus* strain A15-62 was obtained from the Roscoff Culture Collection (http://roscoff-culture-collection.org; RCC strain 2374). It was originally isolated near Cape Verde (Atlantic Ocean) from 30 m depth (29). Both wild-type and mutant strains were routinely grown at 22°C in PCR-S11 (30) in polystyrene flasks (CytoOne, StarLab, USA) at ca. 25 µmol quanta m^{-2} s^{-1} white light. A15-62

mutants were maintained with 50 µg.mL^{-1} kanamycin. Prior to whole-cell absorption and fluorescence measurements as well as to PBS isolation and separation of phycobiliproteins, WT and mutant strains were acclimated for at least two weeks at 20 µE.m^{-2}.s^{-1} BL or GL provided by LED ramps (Luxeon Rebel LED LXML-PB01-0040 and LXML-PM01-0100, respectively; Alpheus, France).

Fluorescence measurements. Spectral properties of WT and mutant strains were measured using a LS-50B spectrofluorimeter (Perkin Elmer, Waltham, MA, USA). Fluorescence excitation was monitored from 420 nm to 560 nm (0.5 nm steps) with emission monitored at 580 nm.

Quantitative PCR Analysis of *mpeW* and *mpeQ* Gene Expression. The expression levels of *mpeQ* and *mpeW* genes was monitored by real time qPCR, as previously described (12). Briefly, triplicate cultures of *Synechococcus* sp. strain A15-62 were acclimated at 20 µmol photons m^{-2} s^{-1} BL or GL then cells were harvested and RNA extracted. For *mpeW* and the control *rnpB* gene, encoding the RNA component of ribonuclease P, we used previously designed primers (12), while for *mpeQ* gene-specific primers were designed using Primer Blast (National Center for Biotechnology Information, Bethesda, MD; Dataset S3). After optimization steps, reverse transcription and qPCR analyses were performed according to (12). The $2^{-\Delta\Delta CT}$ method (31) was finally used to quantify the relative fold change in mRNA levels using *rnpB* as a reference gene to normalize the relative transcript levels.

Plasmid Construction for *mpeW* and *mpeQ* Disruption and Complementation and Construction of Expression Plasmids. Primers are listed in Dataset S3 and plasmids in Dataset S4. The pMUT100-A15-62-mpeW and pMUT100-A15-62-mpeQ constructs were made by PCR amplification of an 820-885 bp internal fragment of A15-62 *mpeW* and A15-62 *mpeQ* using the primers pairs Syn_A15-62_mpeW_126F_BamHI/999R_SphI and Syn_A15-62_mpeQ_123F_EcoRI/ 983R_SphI (*SI Appendix*, Fig. S11).

297 The pMUT100 backbone was PCR-amplified using the primer pair pMUT100_1291F_SphI/5535R_BamHI
298 (pMUT100-A15-62-mpeW) or pMUT100_1291F_SphI/5535R_EcoRI (pMUT100-A15-62-mpeQ). Vector and
299 inserts were digested with SphI and either EcoRI or BamHI (NEB) and ligated. Conjugation between *E. coli*
300 MC1061 containing either pMUT100-A15-62-mpeW or pMUT100-A15-62-mpeQ and *Synechococcus* A15-62
301 was performed as previously described (17, 32). Individual colonies were picked and tested for *mpeW* or
302 *mpeQ* disruption by PCR amplification. For complementation, a derivative of the plasmid pRL153
303 (autonomously replicating in marine *Synechococcus*) was first constructed by cloning a lacZ cassette
304 (pBClacZ) into the BamHI/EagI cloning site of pJS1 for convenient screening of *E. coli* colonies (*SI Appendix*,
305 Fig. S11).

306 The pBClacZ-A15-62-mpeW and pBClacZ-A15-62-mpeQ constructs were made by PCR amplification of
307 the promoter and ribosome-binding site of *mpeW* and *mpeQ*, using the primer pairs Syn_A15-
308 62_unk10_38R_BamHI/Syn_A15-62_fciB_892F_EagI and Syn_A15-62_unk9_13R_ApaI/Syn_A15-
309 62_mpeB_56R, respectively (*SI Appendix*, Fig. S11). PCR fragments were digested with EagI and either BamHI
310 (*mpeW*) or ApaI (*mpeQ*) and ligated into similarly digested pBClacZ. Conjugation between *E. coli* MC1061
311 containing either pBClacZ-A15-62-mpeW or pBClacZ-A15-62-mpeQ and *Synechococcus* A15-62 $mpeW^-$ or
312 $mpeQ^-$ was performed as previously described (17, 32). Individual colonies were picked and tested for *mpeW*
313 or *mpeQ* complementation by PCR amplification (*SI Appendix*, Fig. S11).

314

315 **PBS Isolation and separation of phycobiliproteins.** PBS were purified as previously described (33), starting
316 from 10 L of cultures. All steps were performed at room temperature unless specified. Briefly, cells were
317 harvested by centrifugation, washed twice and resuspended in 0.65M phosphate buffer, and lysed twice
318 using a French press system. Membranes and hydrophobic pigments were removed by 1 h incubation with
319 5% w/v Triton X-100 and centrifugation. The red aqueous layer was loaded onto 0.25-1.0M discontinuous
320 sucrose gradient in phosphate buffer, and centrifuged overnight at 22,500 rpm (range: 42,500-91,300 x *g*) in

a SW28 rotor (Beckman Coulter) at 22°C, or 2-3 h at 49,000 rpm (198,000 *g*) in a VTi 50 rotor (Beckman Coulter). Colored bands were collected and frozen at -20°C until analysis (see Fig. 4 in (34) for illustration).

Purified PBS were dialyzed in 5 mM sodium phosphate buffer, pH 7.0 with 5mM 2-mercaptoethanol (β-Me) and then concentrated by ultrafiltration using Amicon Ultra-15 centrifugal filters (Millipore; Billerica, MA). A sample containing 500 µL of a 1:3 ratio of sample to 9 M urea (pH brought to 2.0 with HCl) was prepared prior to HPLC separation. A Waters E2695 pump (Waters Corporation, Milford, MA) was used in conjunction with a Waters 2996 photodiode array and a Thermo-scientific BioBasic-4 HPLC column (Thermo-scientific, Waltham, MA; 250mm x 4.6 mm, 5 µm particle size) to separate each phycobiliprotein. The program used a flow rate of 1.5 ml min^{-1} with a gradient program starting at a 65:35 ratio of Buffer A (0.1% trifluoroacetic acid solution with 0.001 M sodium azide) to Buffer B (2:1 acetonitrile:isopropanol in 0.1% TFA with 0.001M sodium azide). The starting conditions are 65% A:35% B. After the sample was injected, after holding for 2 min, buffer conditions were ramped to 100% B over 45 min. The samples were monitored at 280 nm, 490 nm, and 550 nm. Separated samples were concentrated using a vacuum centrifuge and stored in the freezer at -20° C until ready for digestion by trypsin.

Heterologous expression and purification of recombinant proteins. For recombinant protein expression, each gene was amplified by PCR from *Synechococcus* RS9916 or A15-62 chromosomal DNA using the primers listed in Dataset S3 and cloned into plasmids as described in Dataset S4. Recombinant proteins were expressed and purified from *E. coli* BL21 (DE3) competent cells (Novagen/EMD Millipore Corp., Darmstadt, Germany) as previously described (35). Once at OD_{600nm}= 0.6, cultures were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), after which the cells were allowed to grow at 18°C for an additional 24 h before being harvested by centrifugation. HT-proteins were purified as described (36).

Protein and bilin analysis by spectroscopy and gel-electrophoresis. Fluorescence emission and absorbance spectra were acquired on a Perkin Elmer LS55 fluorescence spectrometer (Waltham, MA) and a Lambda 35, dual-beam UV/Vis spectrometer (Perkin Elmer) as previously described (37). Polyacrylamide gel electrophoresis (PAGE, 15% w/v) with sodium dodecyl sulfate (SDS) was used to analyze polypeptide samples acquired after purification of proteins as previously described (17). Zn-enhanced fluorescence of covalently attached bilins was visualized using an imaging system (Bio-Rad MP) with excitation at 460 nm (PUB detection) or 540 nm (PEB detection). Proteins in the gels were then stained by incubation in Coomassie brilliant blue G-250 overnight and de-stained in 10 % methanol and 10 % acetic acid and visualized with trans-white light (Bio-Rad MP).

Tryptic digestion and mass spectrometry. Tryptic digestion of proteins was conducted as previously described (38). Tryptic-digested samples were analyzed using LC-MS/MS on an Orbitrap Lumos Fusion mass spectrometer (Thermo Fisher) with an Agilent 1100 Capillary HPLC as its inlet. LC-MS/MS analysis was performed as previously described with a few modifications (13, 17). Alternatively, some samples were reanalyzed using a Thermo Easy-nLC 1000 capillary LC with the following conditions. Buffer A = 0.1% v/v aqueous formic acid; buffer B = 80% v/v acetonitrile, 0.1% v/v formic acid in water. Load 2 µl with 0% B onto a 5 mm long, 0.1 mm i.d. C18 trapping column. A 0.075 mm i.d. x 150 mm long C18 analytical column led directly into the nanospray needle. The gradient was 2% B at 0 min, ramp to 7% B at 0.5 min, ramp to 50% B at 20 min, ramp to 100% B at 21 min, hold 100% B for 9 min. The trap column was re-equilibrated 12 µl of Buffer A and the analytical column with 10 µl of 2% B between each injection. Mass spectra were recorded continuously at 120,000 resolving power. Data dependent tandem mass spectra were recorded at 30,000 resolving power with 3 seconds between MS1 spectra. The tandem mass spectra were processed using Thermo Proteome Discover 2.1 software; a simplified protein database consisting of only the 40 proteins expected to be part of the PBS was used to speed up the analysis. Bilin-containing peptides were confirmed

by manual inspection of their associated MS1, MS2, and UV-VIS spectra. Extracted ion chromatograms (EICs) for each of the bilin-containing peptides were generated using XCalibur 4.0 software (Thermo Fisher). Briefly intensities for a 20-ppm window around the masses indicated were extracted and the resulting chromatograms smoothed with a 5-point boxcar algorithm. Areas were computed using the default Genesis algorithm in XCalibur; a combination of UV-VIS spectra confirmation, retention time and tandem MS/MS data were used to differentiate between the PUB and PEB isomers. The ionizability of PEB and PUB modified versions of the same peptide were considered to be identical for comparisons. The areas of multiple peaks corresponding to the same bilin isomer were added together (oxidized versions of PEB-containing peptides sometimes split into more than one peak with nearly identical tandem mass spectra). The PUB fraction was computed by summing the area of all PUB-containing features for a single modified cysteine and dividing it by the total of both PUB and PEB modified versions of features containing the same cysteine.

ACKNOWLEDGEMENTS. This research was supported by the French national agency for research (ANR) programs CINNAMON (ANR-17-CE2-0014) and EFFICACY (ANR-19-CE02-0019) as well as the EU program Assemble+ for F.P and L.G, by National Science Foundation grants to W.M.S. (MCB-1244339 and MCB-2017171) and to D.M.K. (MCB-1818187 and MCB-2017171) and by a Fulbright Fellowship to T.G. This work was also supported by EMBRC-France, managed by the ANR in the "Investments for the Future" program under the reference ANR-10-INSB-02. We also acknowledge the Ecology and Environment Institute (INEE) of the French National Center for Research (CNRS) for facilitating exchanges between the different partner laboratories through the international project of scientific collaboration (PICS) program ChromaCya (2016-19). The Orbitrap Fusion Lumos was purchased with funds from the Indiana University Precision Health Initiative. The Genomer platform (Biogenouest) is warmly thanked for help with sequencing.

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Legends to Figures

Fig. 1. Phylogeny and similarity of the different members of the MpeQWYZ phycobilin lyase enzyme family and genomic organization of the corresponding genes. (*A*) Comparison of the genomic regions involved in CA4-A and -B processes. The CA4-A genomic island and the PEII sub-region of the PBS genomic region are located at two different loci on the chromosome of *Synechococcus* strain RS9916 (top), whereas in strain A15-62 the CA4-B genomic island is located in the middle of the PBS genomic region, immediately downstream the phycoerythrin-II (PEII) sub-region (bottom). The two genes characterized in the present study (*mpeW* and *mpeQ*) are shown as underlined black bold letters, their functional homologs in the CA4-A strain RS9916 (*mpeY* and *mpeZ*) in black bold letters, and other genes in grey. (*B*) Mean pairwise percentage of identity between the protein sequences of MpeW, MpeQ, MpeZ, and $MpeY^{3a}$ and $MpeY^{3dA}$ (encoded by the two *mpeY* alleles), calculated based on full-length alignments. (*C*) Maximum likelihood phylogeny (protein sequences, LG+I+G+F model) of the MpeQWYZ enzyme family. CpeY sequences were used as outgroup to root the tree. Nodes with bootstrap support >70% and >90% are indicated by empty and filled black dots, respectively. Sequences of A15-62 used in this study are highlighted in bold black letters, other sequences discussed in the text are shown in black, while all other sequences in grey.

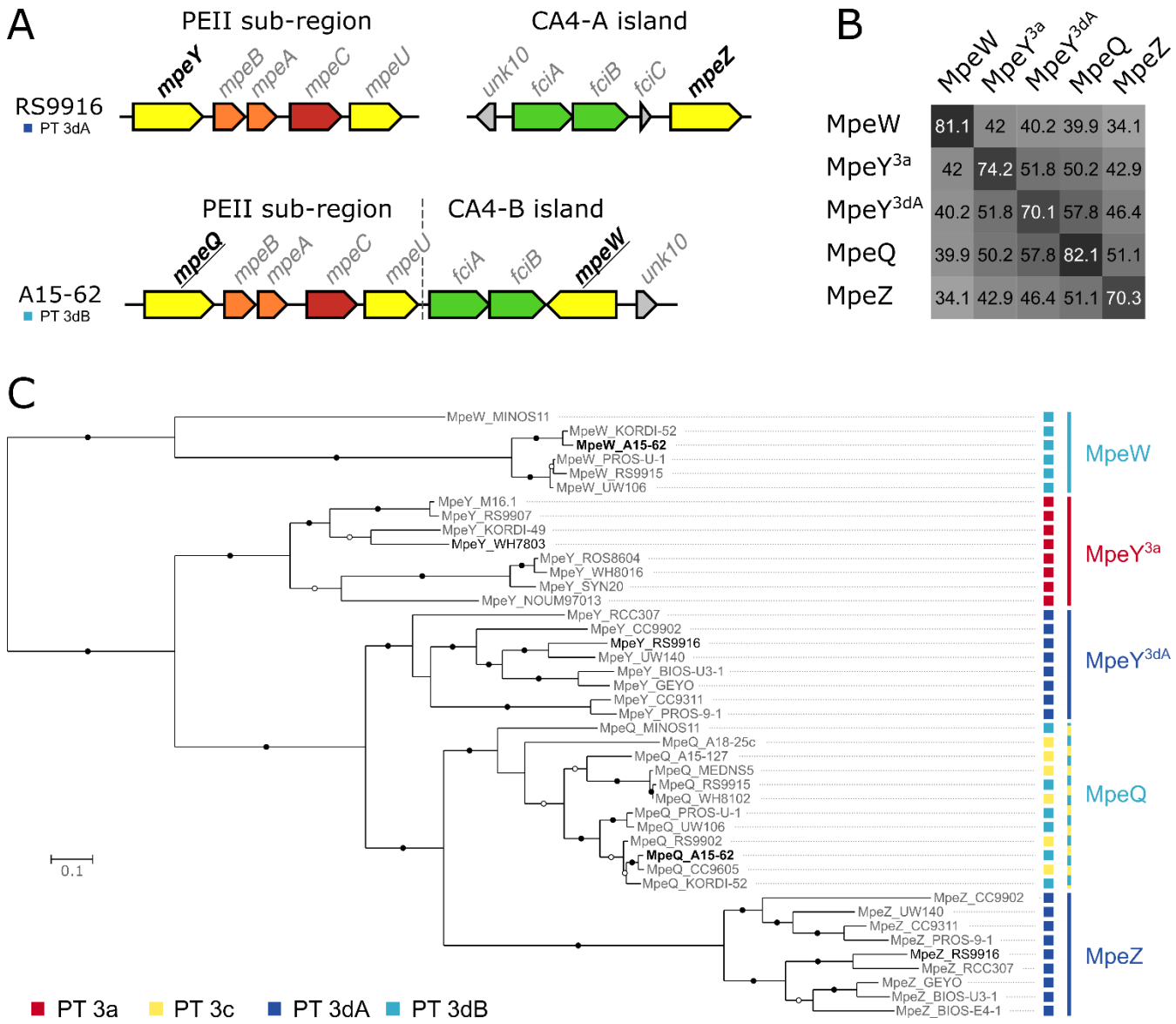
Fig. 2. Absorbance spectra of HPLC-purified phycoerythrin II α-subunit (MpeA) for wild-type (WT) *Synechococcus* sp. A15-62, *mpeW* inactivation mutant (*mpeW*$^-$) and *mpeQ* inactivation mutant (*mpeQ*$^-$) grown under green light (GL) or blue light (BL). (*A* and *C*) WT or mutant cells grown in GL. (*B* and *D*) WT or mutant cells grown in BL. See *SI Appendix*, Fig. S2 for absorbance spectra of the other phycoerythrin-I and II subunits.

Fig. 3. Whole-cell fluorescence excitation spectra for wild-type (WT) *Synechococcus* sp. A15-62 and mutant strains. WT, *mpeW*$^-$ mutant and *mpeW*$^-$ complemented strains grown either in GL (*A*) or BL (*B*). WT, *mpeQ*$^-$ and *mpeQ*$^-$ complemented strains grown either in GL (*C*) or BL (*D*). Fluorescence emission spectra are normalized at 545 nm. Emission was set at 580 nm. Measurements shown here were repeated twice (biological replicates). See *SI Appendix*, Fig. S6 for complementation control.

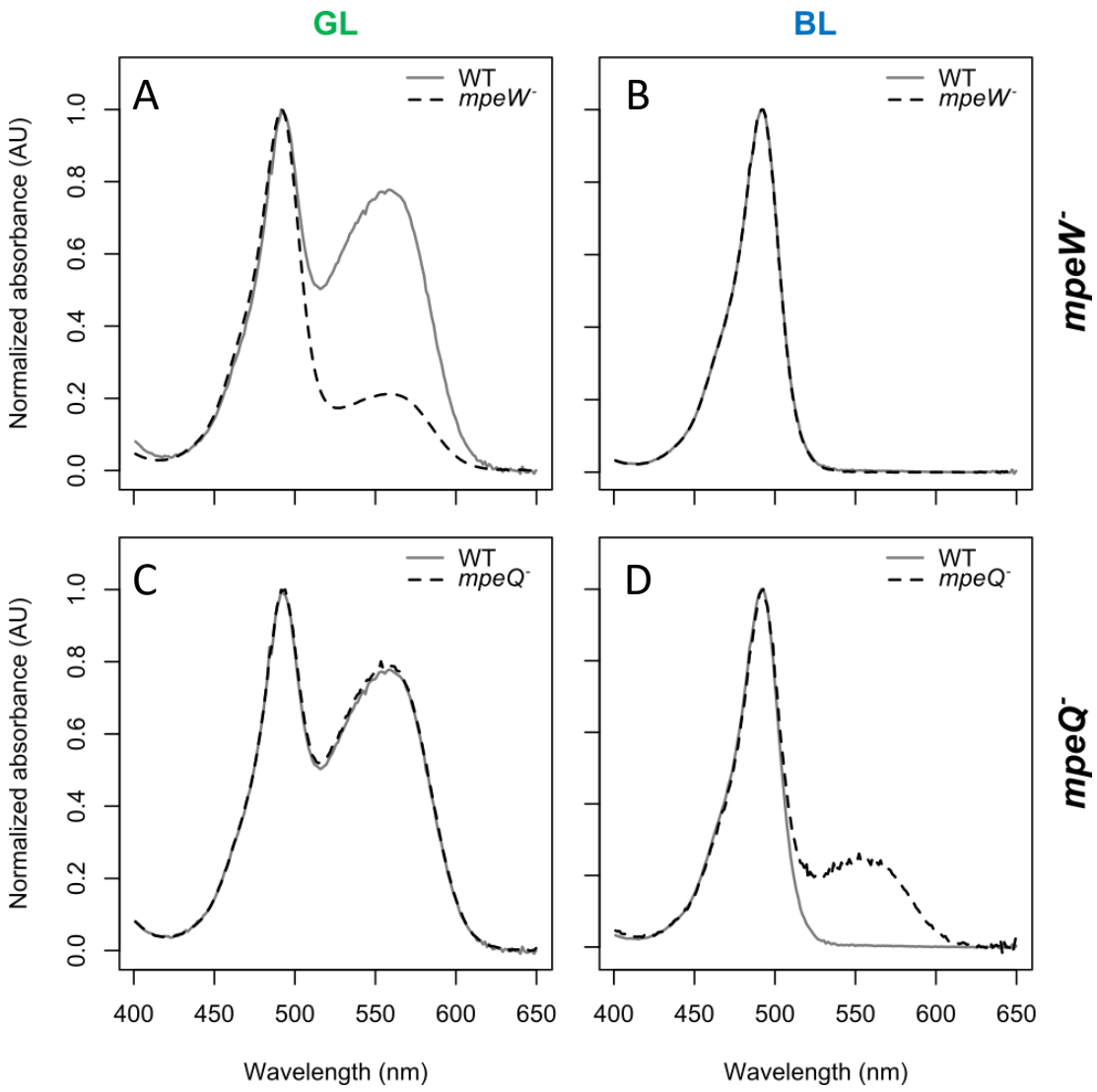
Fig. 4. Determination of the phycobiliprotein subunit and binding site specificities of the MpeW and MpeQ enzymes. Recombinant phycoerythrin-II (PEII) α-subunit (MpeA) from *Synechococcus* sp. A15-62, PEII α- and β-subunits (MpeA, MpeB) and PEI α-subunit (CpeA) from RS9916 as well as site-mutated RS9916 MpeA in which either Cys-83 only or both Cys-75 and Cys-140 were replaced by an alanine [so-called MpeA (C83A) and MpeA (C75A, C140A), respectively], were co-expressed with either MpeQ (*A, B*) or MpeW (*C, D*) from A15-62 along with genes necessary for PEB synthesis. (*A* and *C*) Absorbance (solid lines) and fluorescence emission (dotted lines) spectra of PEB (panel *A*, excitation set at 490 nm) or PUB (panel *C*, excitation set at 440 nm) of purified recombinant WT and site-mutated MpeA from RS9916. The secondary emission peak at 610 nm in panel *A* likely corresponds to PEB at C83 (see text). (*B* and *D*) Zinc-enhanced fluorescence of SDS-PAGE gel showing relative PEB or PUB covalently bound to different recombinant phycoerythrin subunits under various coexpression conditions (indicated on top) for MpeW (*B*) or MpeQ (*D*). Data shown here are representative of three technical replicates.

Table 1: Percentage of PUB chromophores, expressed as a PUB/(PEB+PUB) ratio, found at the different cysteinyl sites of the PEI and PEII α- (CpeA, MpeA) and β-subunits (CpeB, MpeB) in *Synechococcus* sp. A15-62 wild type cells and *mpeW*⁻ and *mpeQ*⁻ mutants grown under GL and BL. Underline indicate that the chromophorylation was confirmed by LC-MS/MS mass spectrometry. Bold indicate that the chromophore is expected to change during the CA4 process. At some sites, the mass spectrometry analysis detected a mix of PUB and PEB and the percentage of PUB (%PUB) is indicated. Abbreviations: BL, blue light; GL green light; ND, not determined; PBP, phycobiliprotein; PEB, phycoerythrobilin; PEI, phycoerythrin I; PEII, phycoerythrin II; PUB, phycourobilin; WT, wild-type.

PBP	Subunit	Cysteine position	%PUB attached in GL			%PUB attached in BL		
			WT	*mpeW*⁻	*mpeQ*⁻	WT	*mpeW*⁻	*mpeQ*⁻
PEII	MpeA	75	100	100	100	100	100	100
		83	**9**	**99**	**2**	**100**	**99**	**14**
		140	**15**	**35**	**36**	**93**	**99**	**99**
PEII	MpeB	50,61	100	ND	ND	100	ND	ND
		82	0	ND	ND	0	ND	ND
		165	0	ND	ND	0	ND	ND
PEI	CpeA	82	0	0	0	0	0	0
		139	**0**	**100**	**21**	**100**	**98**	**43**
PEI	CpeB	50,61	100	ND	ND	100	ND	ND
		82	0	ND	ND	0	ND	ND
		159	0	ND	ND	0	ND	ND



Grébert *et al.*, Figure 1



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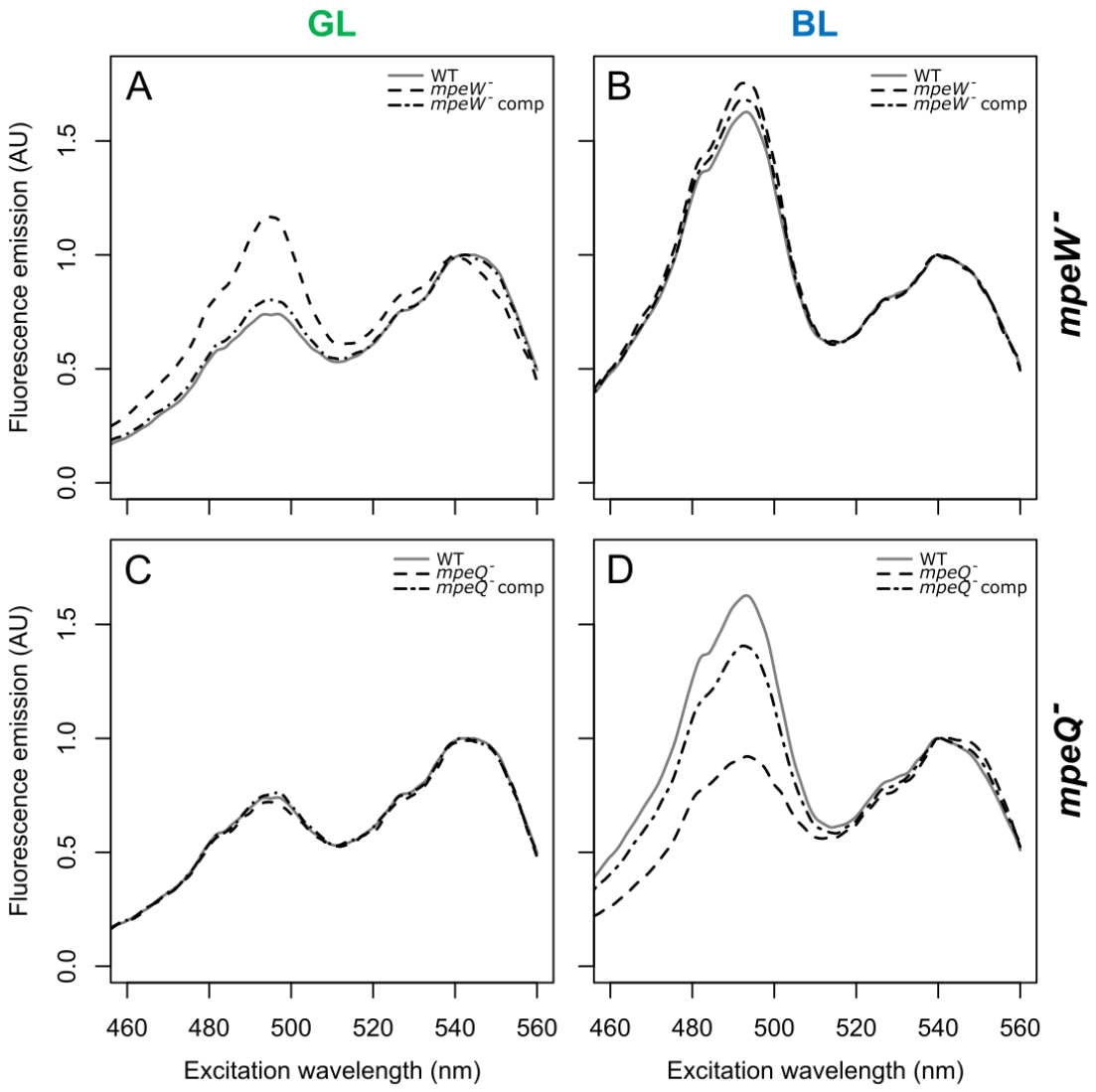

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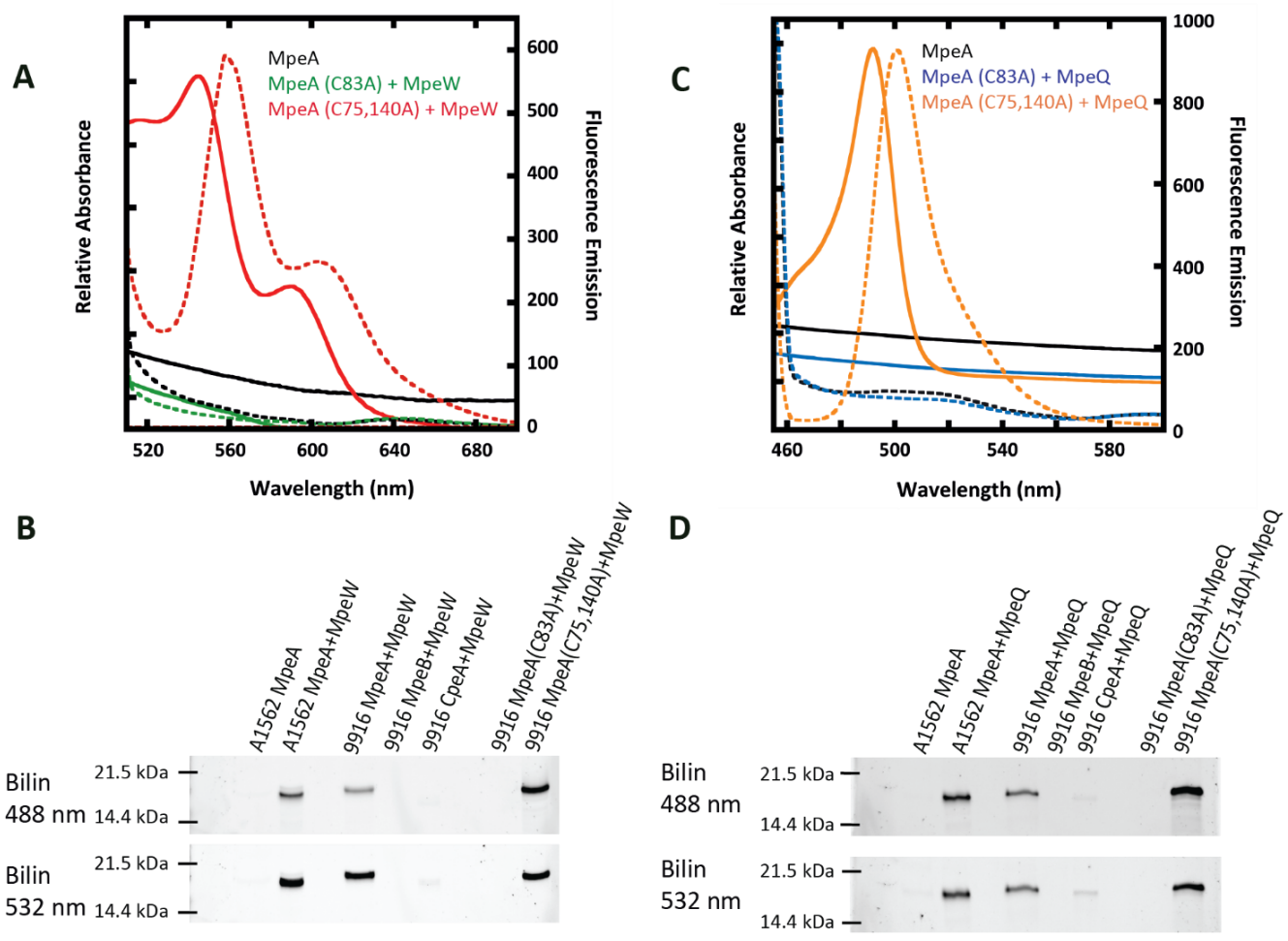

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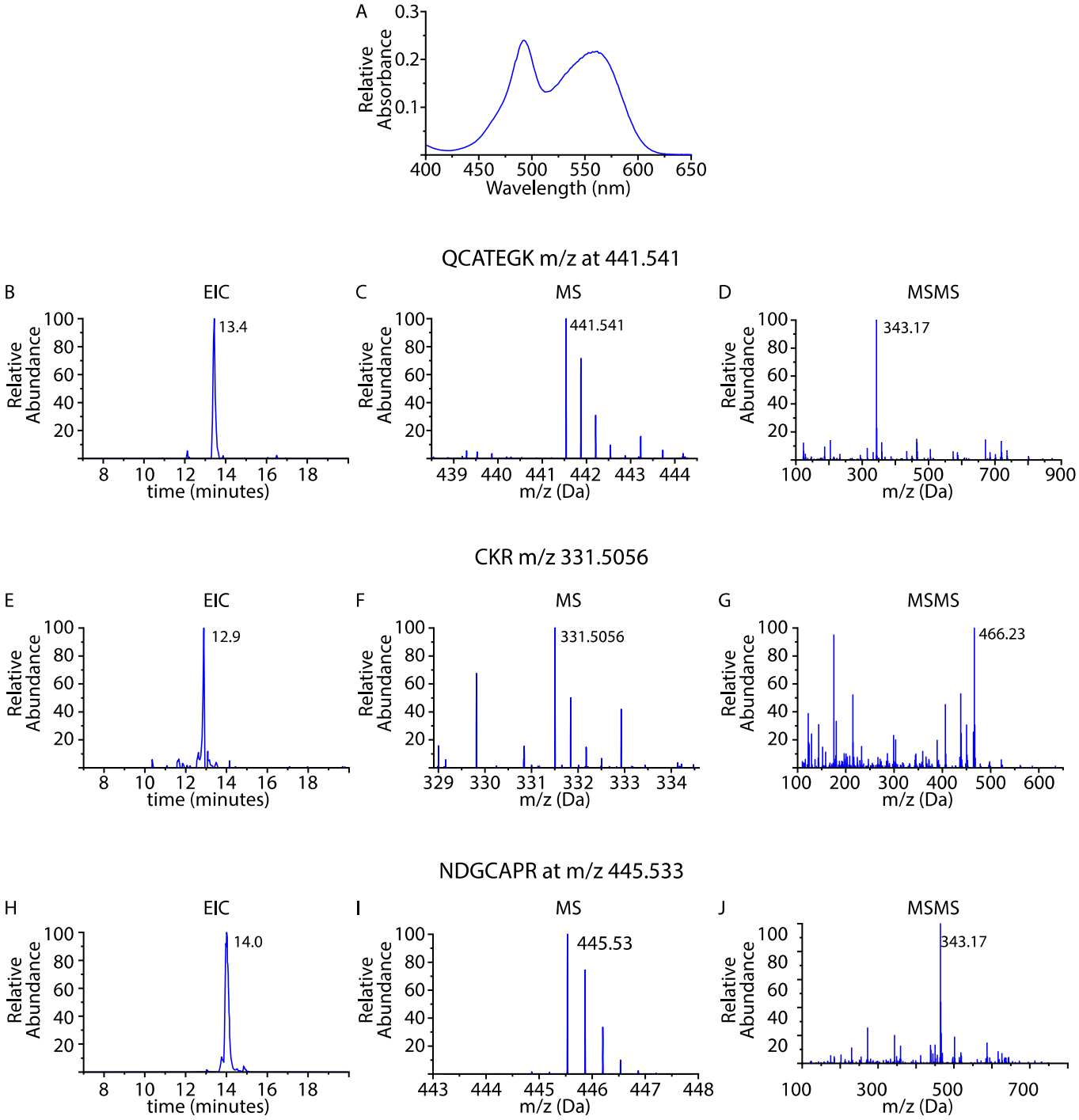


Fig. S1. Absorption spectrum and mass spectrometry analysis of bilin chromophorylation of *Synechococcus* sp. WH7803 phycoerythrin-II α-subunit (MpeA). (*A*) UV-VIS spectrum from the purified *Synechococcus* WH7803 MpeA showing absorbance peaks at 490 and 550 nm, indicating that the protein is binding both PUB and PEB. (*B*) LC-MS analysis showing the EIC at mass 441.541 for the bilin-modified peptide QC*$_{75}$ATEGK showing an elution peak at 13.4 min. (*C*) MS scan of the elution at 13.4 min showing the isotope profile of a peptide with m/z 441.541. (*D*) Tandem mass spectrum of m/z 441.5410 at 13.4 min. The relatively early elution and strong diagnostic ion at m/z 343.17 indicate that it binds with PUB. (*E*) LC-MS analysis showing the EIC at mass 331.506 for the bilin-modified peptide C*$_{83}$KR showing primarily a single species eluting at 12.9 min. (*F*) The MS scan confirms the expected mass at 331.506. (*G*) The strong relative abundance of the diagnostic ion at m/z 466.23, demonstrated the presence of PEB. (*H*) LC-MS analysis showing the EIC at mass 445.533 for the bilin-modified peptide NDGC*$_{140}$APR showing an elution peak at 14.0 min. (*I*) MS scan of the elution at 14.0 min showing the isotope profile of a peptide with m/z 445.533. (*J*) Database interpretation of the MS/MS fragmentation spectra from the 445.533 peptide confirmed the sequence as NDGC*$_{140}$APR. The relatively early elution and strong diagnostic ion at m/z 343.17 indicate modification with PUB. Abbreviations: EIC, extracted ion chromatograph; LC, liquid chromatography; MS, mass spectrometry; PEB, phycoerythrobilin; PUB, phycourobilin.

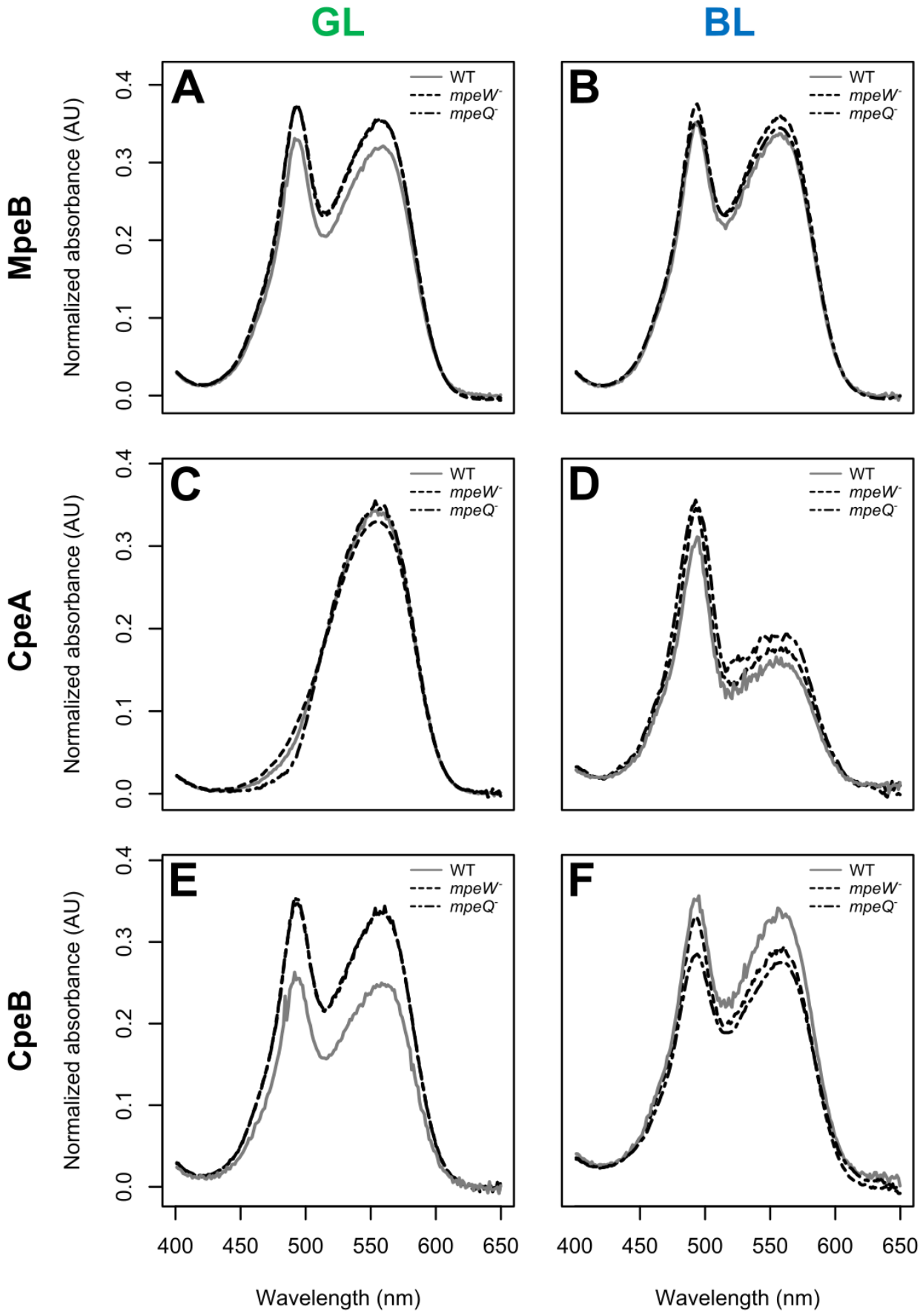


Fig. S2. Absorbance spectra of HPLC-purified phycoerythrin-II β-subunit (MpeB), phycoerythrin-I α- (CpeA) and β- (CpeB) subunits of *Synechococcus* sp. A15-62 WT, *mpeW* inactivation mutant (*mpeW*$^-$) and *mpeQ* inactivation mutant (*mpeQ*$^-$) cells grown under GL or BL. (*A*, *C* and *E*) WT or mutant grown in GL. (*B*, *D* and *F*) WT or mutant grown in BL. (*A* and *B*) MpeB. (*C* and *D*) CpeA. (*E* and *F*) CpeB. See Fig. 2 for absorbance spectra of MpeA. Abbreviations: BL, blue light; GL, green light.

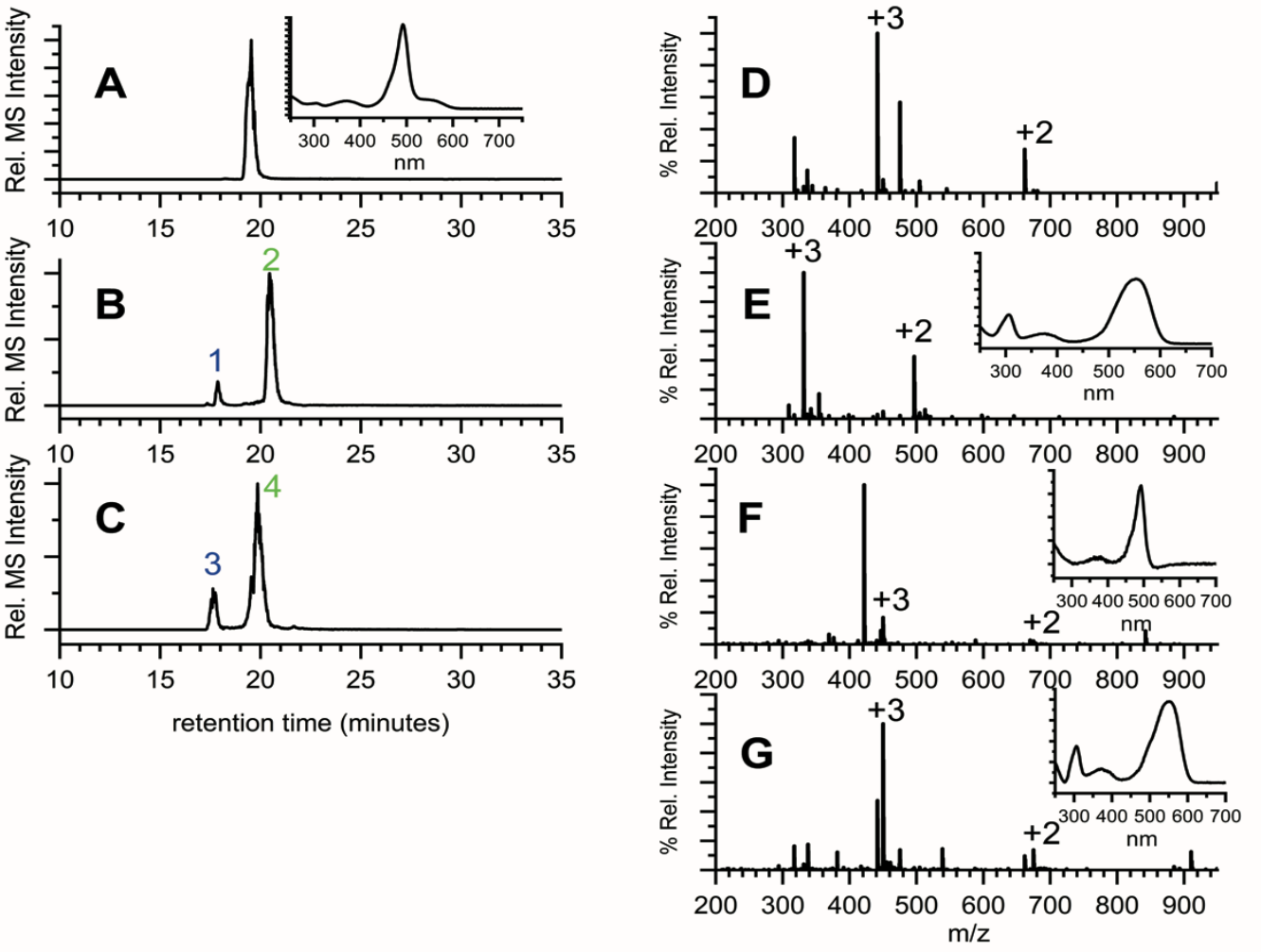


Fig. S3. Mass spectrometry analysis of phycoerythrin-II α-subunit (MpeA) isolated from *Synechococcus* sp. A15-62 wild-type cells grown in GL. (*A*) EIC for m/z 441.5532, $KC^*_{75}ATEGK^{3+}$ where the * corresponds to a Cys modified by PUB. (*B*) EIC for m/z 336.8372, $C^*_{83}KR^{3+}$, where the * corresponds to Cys modified by PUB (peak 1) or PEB and oxidation (peak 2). (*C*) EIC for m/z 450.2204, $SRGC^*_{140}APR$, where the * corresponds to Cys modified by PUB (peak 3) or PEB and oxidation (peak 4). (*D*) MS data for the 19.5 min peak in panel *A*. (*E*) MS data for peak 2 in panel *B;* inset UV-VIS spectrum at 19.5 min indicates PEB on Cys_{140}. (*F*) MS from 17.60 min peak 3 in C; UV-VIS spectrum indicates PUB modified Cys. (*G*) MS data for the 19.9 min peak 4 in panel *C*; UV-VIS indicates PEB modified Cys. The charge states refer to $(peptide+nH)^{n+}$ ions. Abbreviations: EIC, extracted ion chromatograph; GL, green light; MS, mass spectrometry.

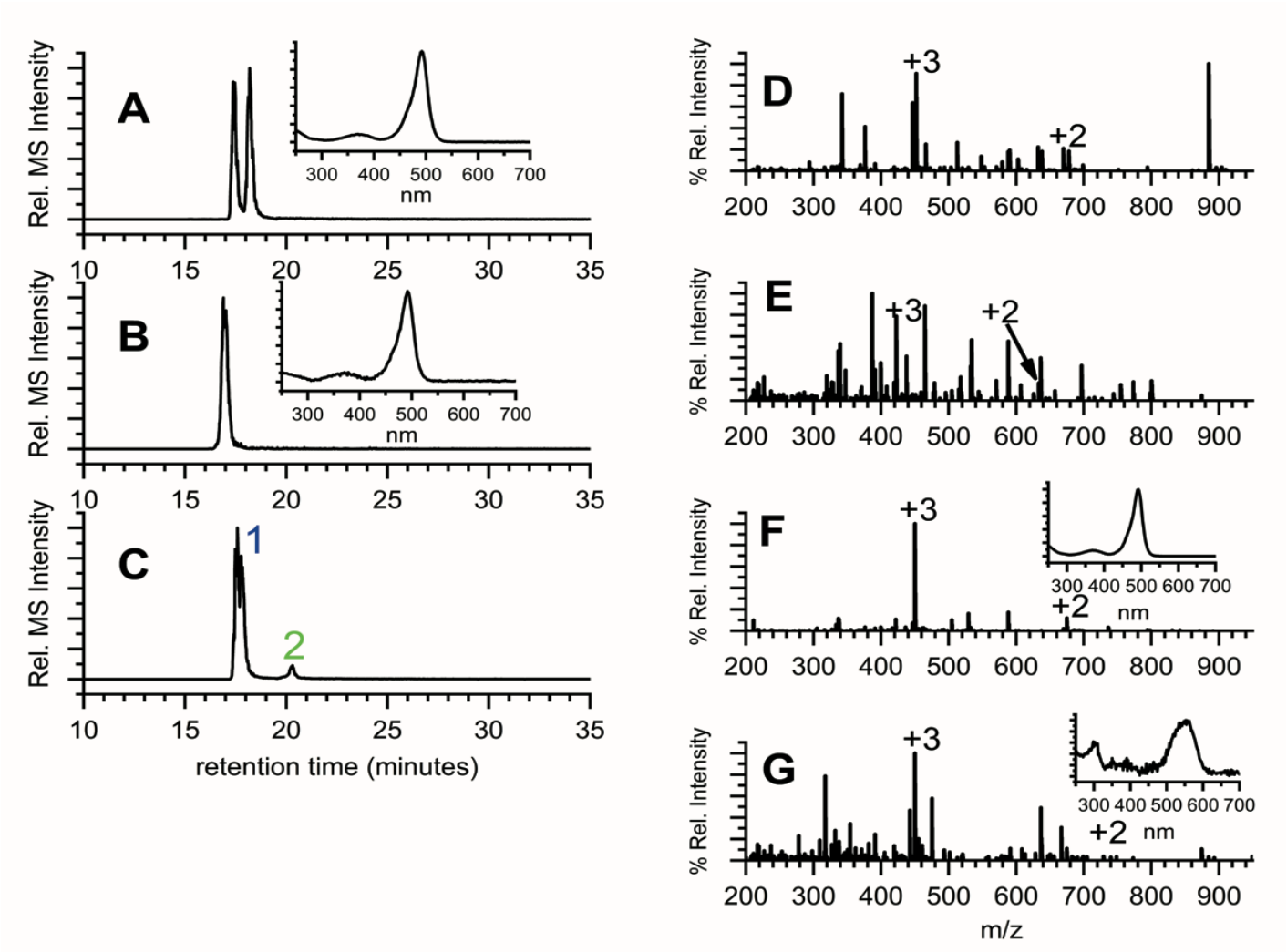


Fig. S4. Mass spectrometry data for phycoerythrin-II α-subunit (MpeA) isolated from *Synechococcus* sp. A15-62 wild-type cells grown in BL. (*A*) EIC for m/z 446.8848, $KC^*_{75}ATEGK^{3+}$, where the * corresponds to Cys modified by PUB and oxidation. (*B*) EIC for m/z 422.5498, $EKC^*_{83}KR^{3+}$. (*C*) EIC for m/z 450.2204, $SRGC^*_{140}APR$. (*D*) MS data for the 18.20 min peak in panel *A* (17.39 min peak in panel *A* gave similar MS, MS-MS, and UV-VIS spectra). (*E*) MS data for the 16.90 min peak in panel *B*. (*F*) MS data for the 17.60 min peak in panel *C* (peak 1); UV-VIS spectrum indicates PUB modified Cys. (*G*) MS data for the 20.31 min peak in panel *C* (peak 2); UV-VIS indicates PEB modified Cys. The charge states indicated in the MS refer to $(peptide+nH)^{n+}$ ions. Abbreviations: BL, blue light; MS, mass spectrometry; EIC, extracted ion chromatograph.

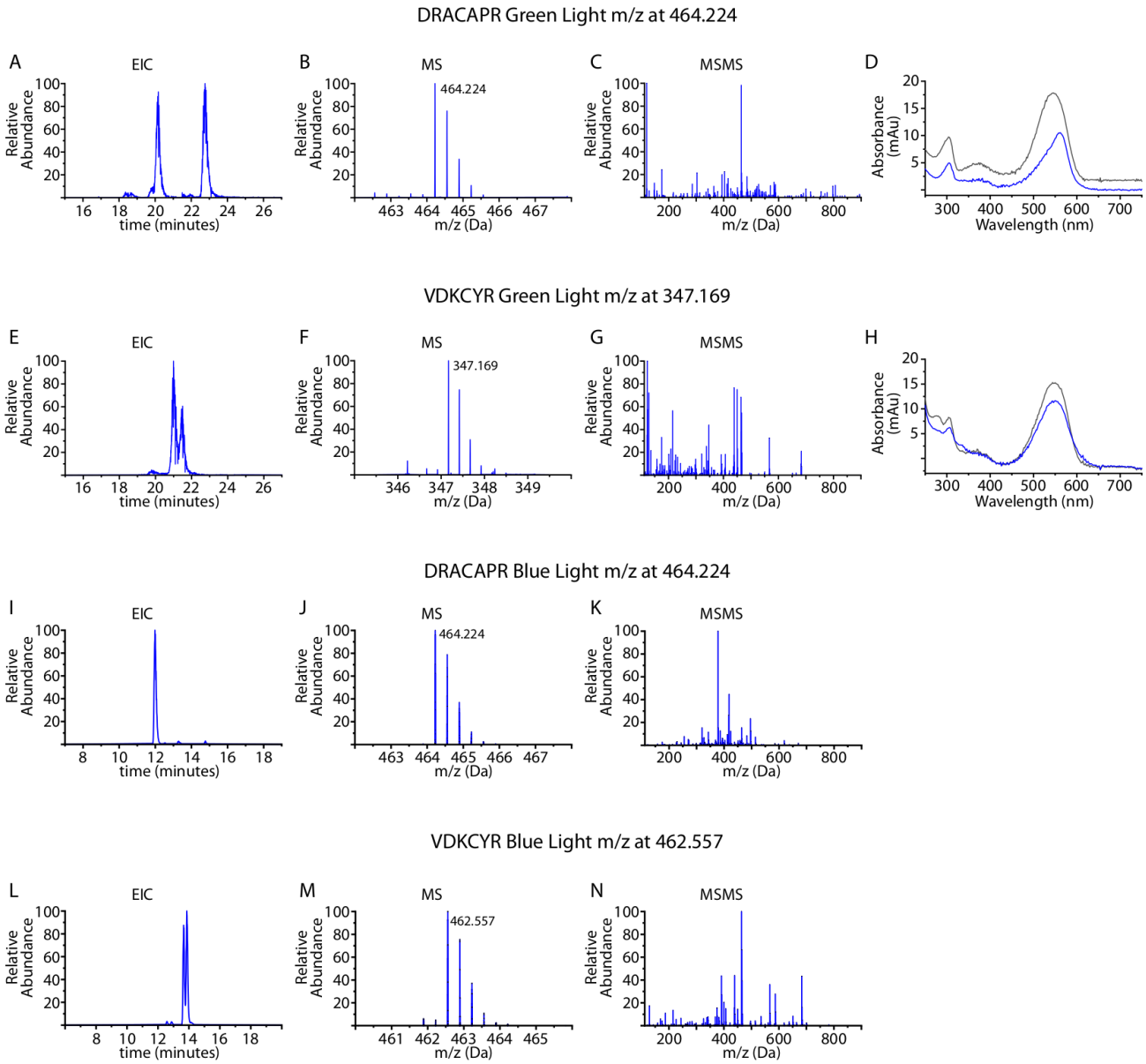


Fig. S5. Tandem mass spectrometry data for phycoerythrin-I α-subunit CpeA-C82 and C139 isolated from for A15-62 WT cells grown in GL and BL. (*A*) LC-MS profile showing the EIC at mass 464.224 for the oxidized version of the bilin-modified peptide DRAC*$_{139}$APR grown in GL with a doublet eluting at 20.1 and 22.8 min. (*B*) MS scan of the elution at 20.1 min showing the isotope profile of a peptide with the expected m/z of 464.224. (*C*) Database interpretation of the MS-MS fragmentation spectra from this 464.224 peptide confirmed the sequence as DRAC*$_{139}$APR. (*D*) UV absorbance spectra at 20.1 min (black) and 22.8 min (blue), with absorbance maxima at 550 nm, indicating both species are modified with PEB. (*E*) LC-MS profile showing the EIC at mass 347.169 for the oxidized version of the bilin-modified peptide VDKC*$_{82}$YR grown in GL with a doublet eluting at 20.1 and 21.1 min. (*F*) MS scan of the elution averaged from 20.9 to 21.2 min showing the isotope profile of a peptide with the expected m/z of 347.169. (*G*) Database interpretation of the MS/MS fragmentation spectra from this 347.2 peptide confirmed the sequence as VDKC*$_{82}$YR. (*H*) UV absorbance spectra at 20.1 min (black) and 21.1 min (blue), with absorbance maxima at 550 nm, indicating that both species are modified with PEB. (*I*) LC-MS profile showing the EIC at mass 464.224 for the oxidized version of the bilin-modified peptide DRAC*$_{139}$APR grown in blue light eluting at 11.88 min. (*J*) MS scan of the elution at 11.88 min showing the isotope profile of a peptide with the expected m/z of 464.224. (*K*) Database interpretation of the MS/MS fragmentation spectra from this 464.224 peptide confirmed the sequence as DRAC*$_{139}$APR. (*L*) LC MS showing the EIC at mass 462.557 for the bilin-modified peptide VDKC*$_{82}$YR grown in BL with a peak eluting in a doublet at 13.65 and 14.17 min. (*M*) MS scan of the elution at 13.65 min showing the isotope profile of a peptide with the expected m/z of 462.557. (*N*) Database interpretation of the MS/MS fragmentation spectra from this 462.557 peptide confirmed the sequence as VDKC*$_{82}$YR. This sample was run without UV/VIS collection, but retention time and tandem MS-MS data from other samples with UV/VIS data were used to differentiate between the PUB and PEB isomers of these peptides. Abbreviations: BL, blue light; EIC, extracted ion chromatograph; GL, green light; LC, liquid chromatography; MS, mass spectrometry; PEB, phycoerythrobilin; PUB, phycourobilin.

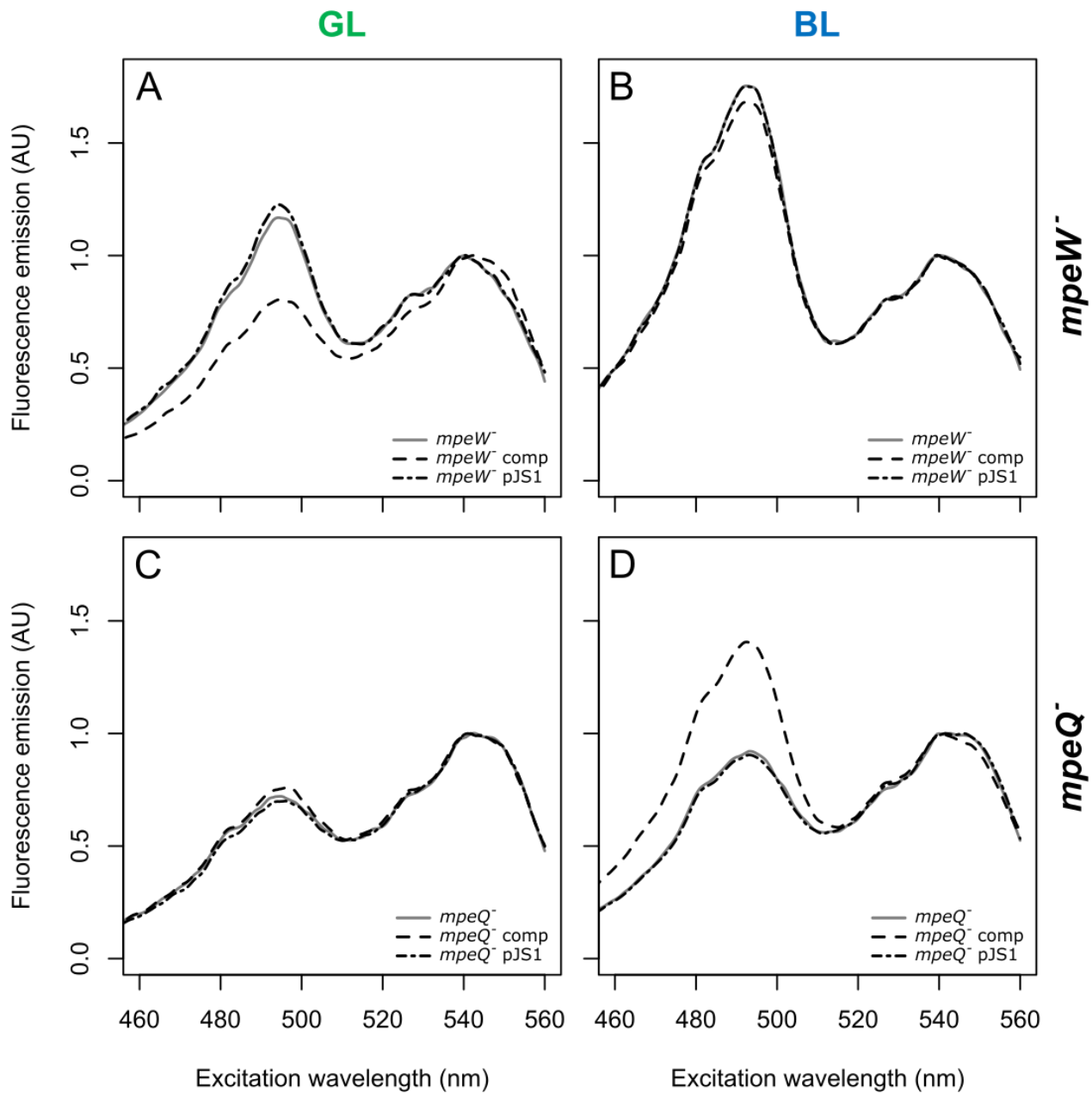


Fig. S6. Whole-cell fluorescence excitation spectra for *Synechococcus* sp. A15-62 mutant and complemented strains. The *mpeW* inactivation mutant (*mpeW*$^-$), *mpeQ* inactivation mutant (*mpeQ*$^-$), complementation strains *mpeW*$^-$ comp (*mpeW*$^-$ mutant with plasmid pTG_A15-62_mpeW_comp) and *mpeQ*$^-$ comp (*mpeQ*$^-$ mutant with plasmid pTG_A15-62_mpeQ_comp) as well as complementation controls *mpeW*$^-$ pJS1 and *mpeQ*$^-$ pJS1 (*mpeW*$^-$ and *mpeQ*$^-$ mutants with empty plasmid pJS1) were acclimated for at least two weeks in either green light (GL) or blue light (BL) before the measurement. (*A, B*) *mpeW*$^-$ mutant, complemented and complementation control strains. (*C, D*) *mpeQ*- mutant, complemented and complementation control strains. Fluorescence emission spectra are normalized at 545 nm for panels *A* and *B* and at 495 nm for panels *C* and *D*. Emission was set at 580 nm. Measurements were repeated twice (biological replicates).

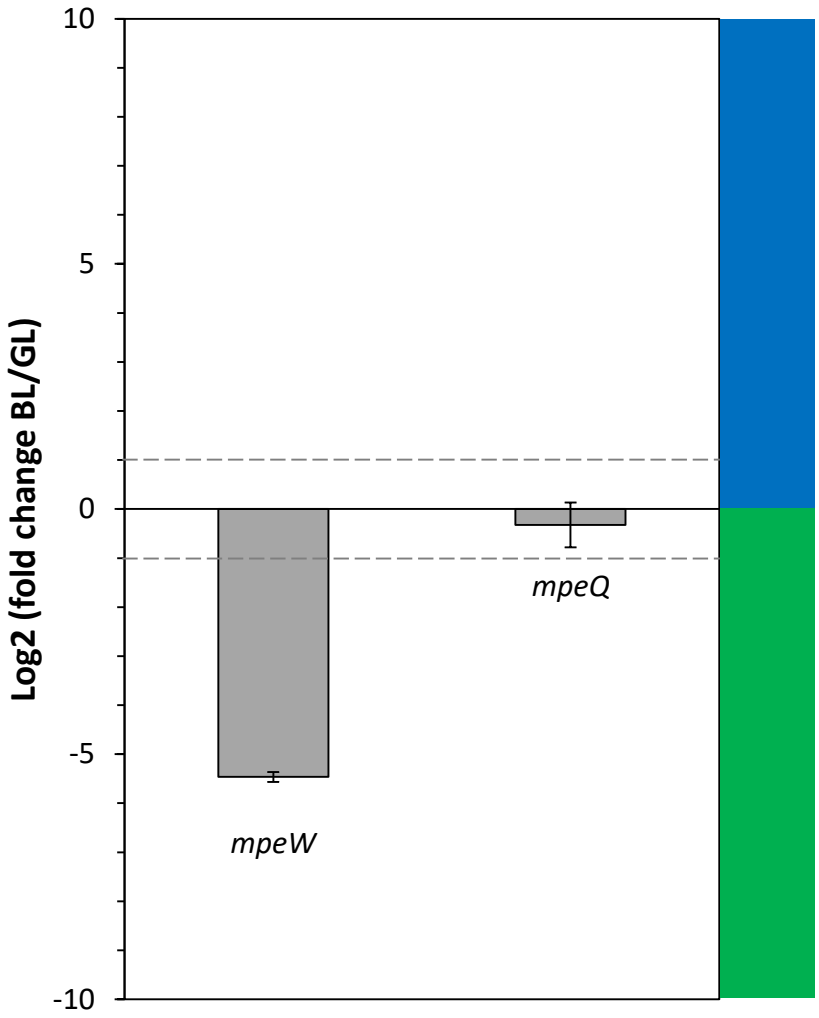


Fig. S7. Differential expression of *mpeW* or *mpeQ* genes in WT *Synechococcus* sp. A15-62 cultures acclimated to 20 µmol photons m^{-2} s^{-1} blue and green light, as measured by real time PCR. Mean and standard deviation were calculated from three biological replicates. Only differential transcript levels above or below the dotted lines (log2(FC) > 1 or < -1) are significant.

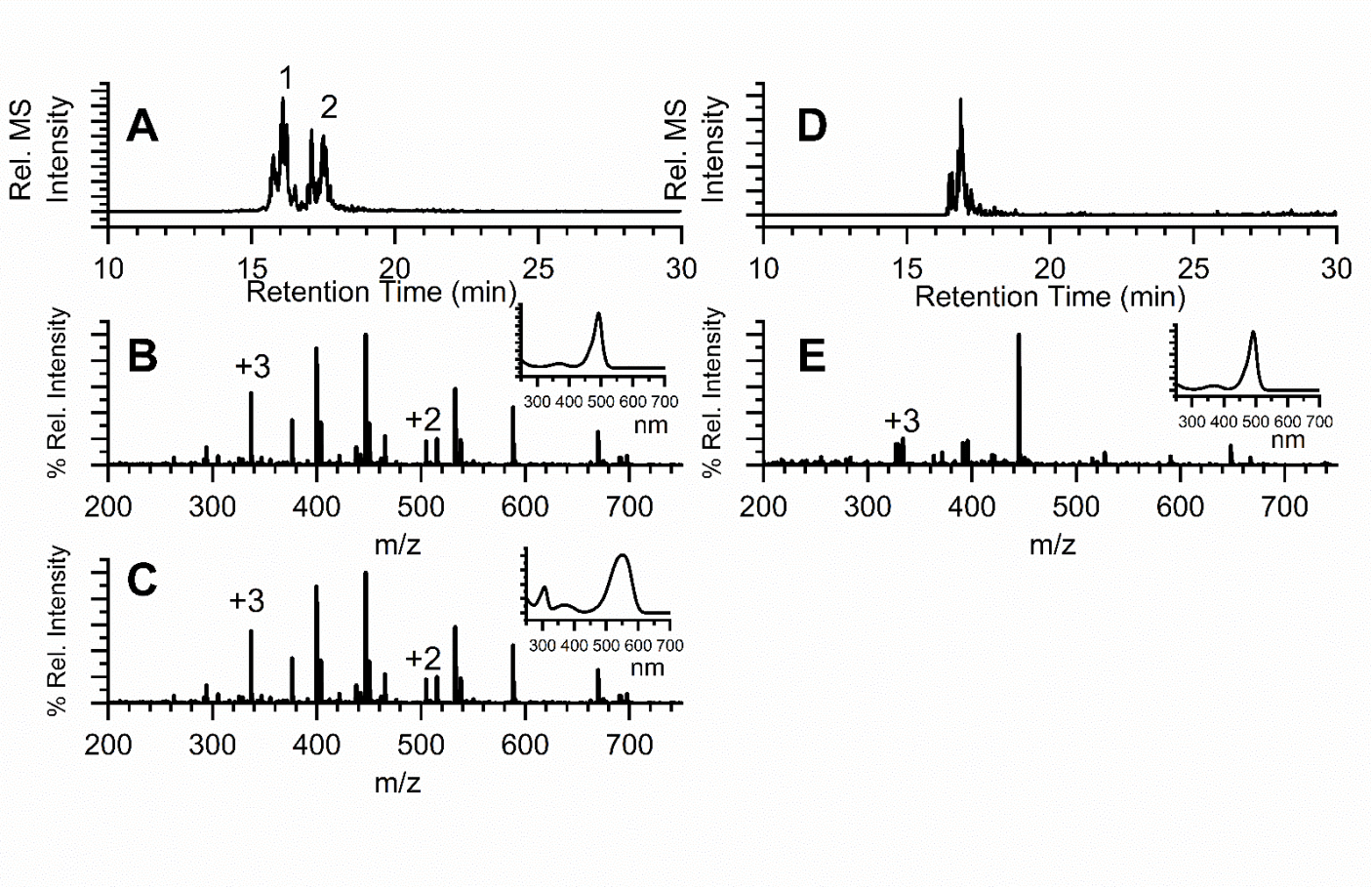


Fig. S8. Mass spectrometry analyses of the phycoerythrin-II α-subunit MpeA-C83 peptides isolated from *Synechococcus* sp. A15-62 *mpeQ*$^{-}$ mutant cells grown in GL and BL. (*A*) EIC for C*$_{83}$KR+bilin+oxygen (m/z 336.8372^{3+}) in GL. (*D*) EIC for C*$_{83}$KR+bilin (m/z 331.5056^{3+}) in BL. (*B*) Mass spectra and UV-VIS spectra (insert) foe the 16.1 min peak (peak 1 in panel *A*). (*C*) Mass spectra and UV-VIS spectra (insert) for the 17.5 min peak (peak 2 in panel *A*). (*E*) Mass spectra and UV-VIS spectra (insert) for the 16.9 min peak in panel (*D*). Charges labeled in mass spectra indicate peaks arising from C*$_{83}$KR peptides. Abbreviations: BL, blue light; EIC, Extracted ion chromatograms; GL, green light.

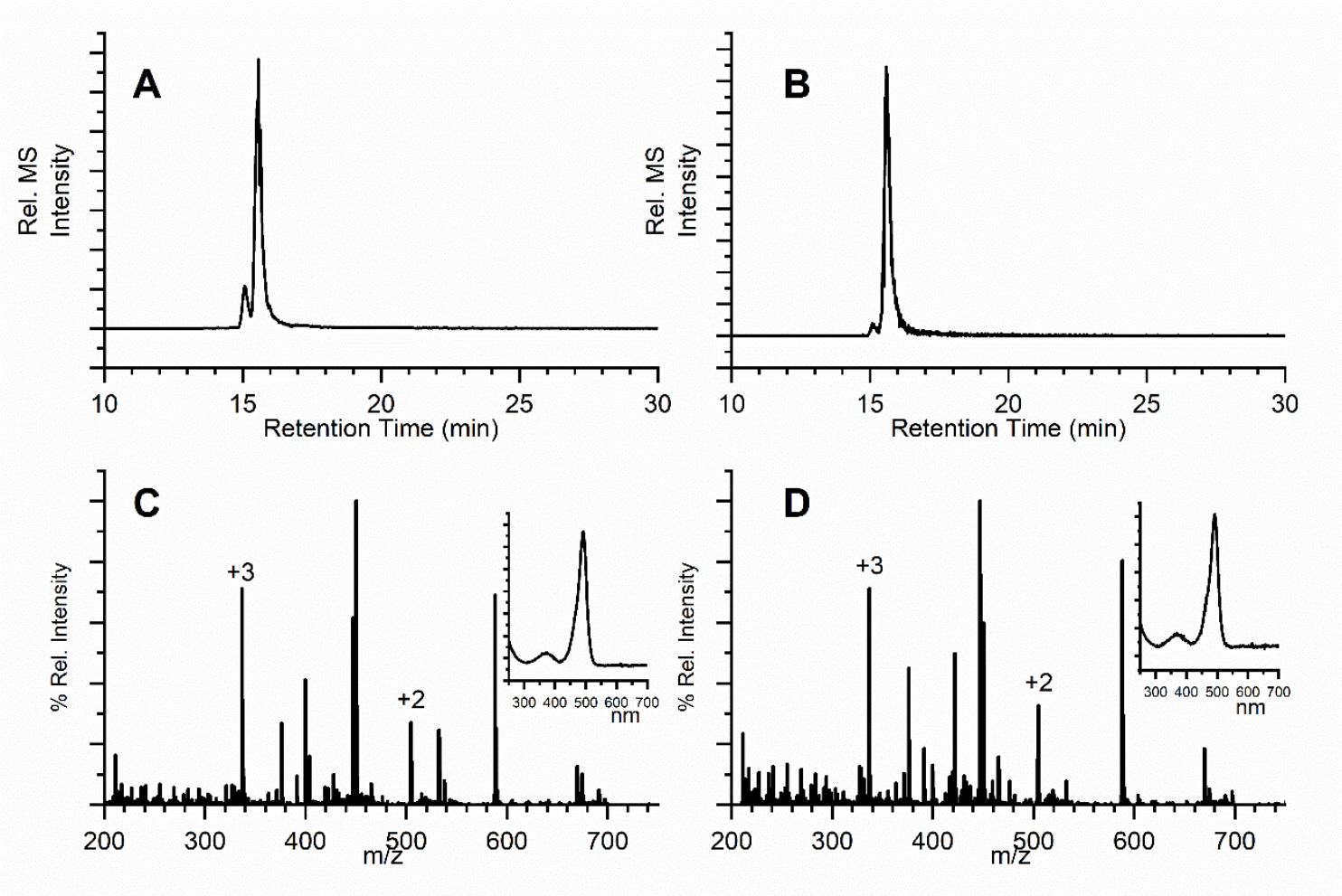


Fig. S9. Mass spectrometry analyses of the phycoerythrin-II α-subunit MpeA-C83 peptides isolated from *Synechococcus* A15-62 *mpeW*$^{-}$ mutant cells grown in GL and BL. EIC for C*$_{83}$KR+bilin+oxygen (m/z 336.8373^{3+}) grown in (*A*) BL and (*B*) GL. (*C-D*) Mass spectra and UV-VIS spectra (insert) from 15.6 min peak in panels *A* and *B*, respectively. Both peptides were shown to contain PUB. BL, blue light; EIC, Extracted ion chromatograms; GL, green light.

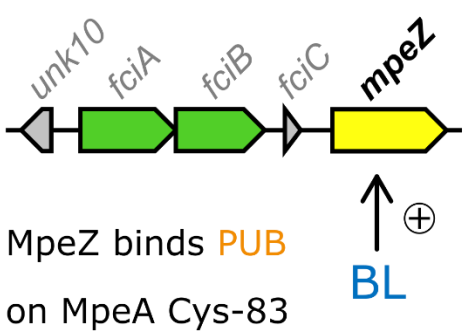
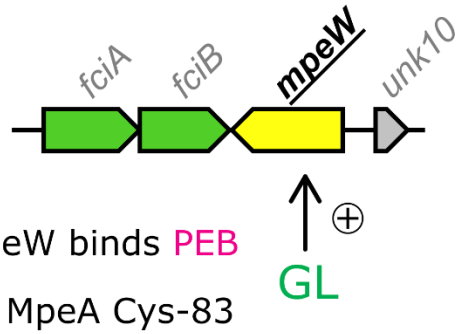
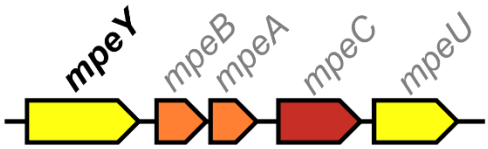
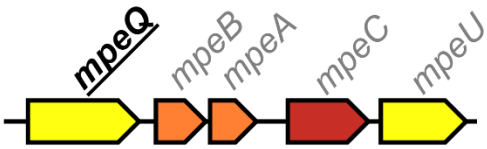
Model strain	RS9916	A15-62
Clade	IX	IIc
Pigment type	3dA	3dB
CA4 island	CA4-A  	CA4-B 
PBS region	 MpeY binds PEB on MpeA Cys-83	 MpeQ binds PUB on MpeA Cys-83
References	(1, 2)	This study

Fig. S10. Summary of the differences between the two types of chromatic acclimation (CA4-A and –B) occurring in marine *Synechococcus*. The table summarizes the main characteristics of the model strains and recapitulates the results from two previous studies on CA4-A using RS9916 and how they compare to the new results obtained in the present study on CA4-B using A15-62. The 'PBS region' refers to the genomic region that gathers most genes involved in the biosynthesis and regulation of phycobilisome rods (3). Phylogenetically, both strains belong to sub-cluster 5.1 but to different (sub)clades *sensu* (4).

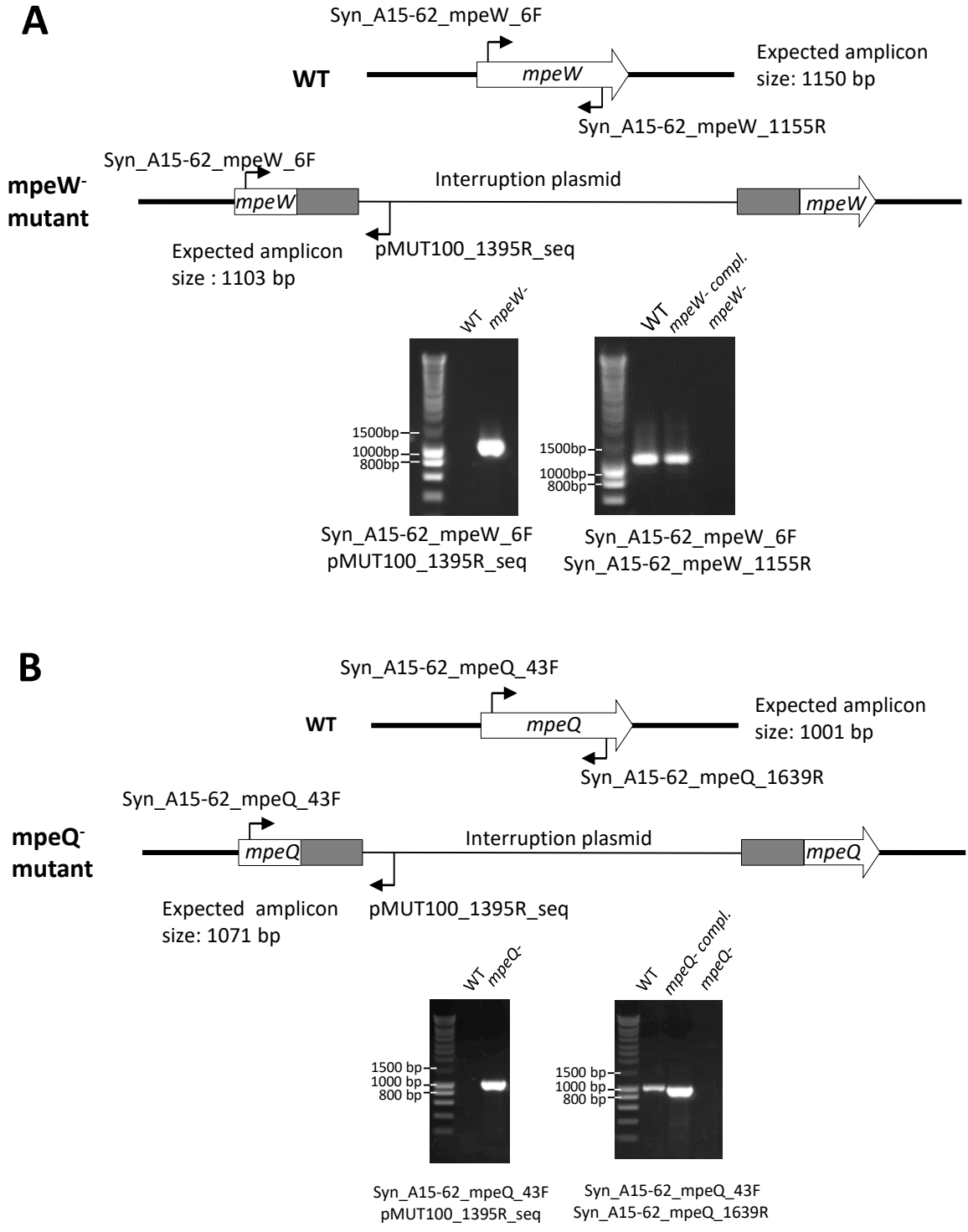


Fig. S11. Constructions of *mpeW*$^{-}$*/Q*$^{-}$ mutants and complementations. (*A*) Scheme of *mpeW* interruption and PCR verification of non-complemented and complemented *mpeW*$^{-}$ mutant. (*B*) Scheme of *mpeQ* interruption and PCR verification of non-complemented and complemented mutant *mpeQ*$^{-}$ mutant.

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1. G. K. Farrant, *et al.*, Delineating ecologically significant taxonomic units from global patterns of marine picocyanobacteria. *Proc. Natl. Acad. Sci.*, 201524865 (2016).

2. J. E. Sanfilippo, *et al.*, Interplay between differentially expressed enzymes contributes to light color acclimation in marine *Synechococcus*. *Proc. Natl. Acad. Sci.* **116**, 6457–6462 (2019).

3. A. Shukla, *et al.*, Phycoerythrin-specific bilin lyase-isomerase controls blue-green chromatic acclimation in marine *Synechococcus*. *Proc. Natl. Acad. Sci.* **109**, 20136–20141 (2012).

4. C. Six, *et al.*, Diversity and evolution of phycobilisomes in marine *Synechococcus* spp.: a comparative genomics study. *Genome Biol* **8**, R259 (2007)