



Genomic analysis highlights a novel class of DPCF-type cyanobacteriochrome

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Abstract

Cyanobacteriochromes (CBCRs) are light-sensitive photoreceptors that regulate diverse cyanobacterial responses to light. In this study, we combined large-scale genome mining, phylogenetics, heterologous expression, structural modeling, and spectroscopy to identify and characterize a previously unrecognized family of CBCR photoreceptors. CBCRs are light-sensing proteins that bind a chromophore through a GAF (cGMP-specific phosphodiesterases, adenylyl cyclases, and the bacterial transcription factor FhlA) domain. This family is defined by a conserved N-terminal DUF4118 domain (Domain of Unknown Function 4118) and a DPCF (Asp-Pro-Cys-Phe) motif-containing GAF domain. Interestingly, these GAF domains are phylogenetically much closer to recently described DPCI-2 (Asp-Pro-Cys-Ile) GAF domains than to conventional DPCF (Asp-Pro-Cys-Phe) GAF domains, therefore named as DPCF-2 GAF domains. These proteins, primarily distributed within the Nostocales order, are consistently associated with conserved two-component signaling (TCS) elements, suggesting their integration into light-regulated signaling cascades that might coordinate transcriptional responses in filamentous cyanobacteria. Spectral characterization of the DPCF-2 GAF domain from *Tolypothrix* sp. PCC 7910 revealed a stable, fully reversible blue-teal photocycle, shifting from a blue-absorbing dark state (P_b, 417 nm) to a teal-absorbing state (P_t, 514 nm) upon blue light exposure. This distinctive spectral response opens new avenues for exploring both the biological functions of DPCF-2 CBCRs and their potential biotechnological applications.

Keywords Cyanobacteriochrome · DUF4118 domain · GAF domain · Blue-teal photocycle · Two-component system

1 Introduction

Cyanobacteria are prokaryotic microbes that perform oxygenic photosynthesis and must adapt to varying light conditions for survival and growth. To optimize light-mediated processes, they possess a diverse array of photoreceptors

known as cyanobacteriochromes (CBCRs) [1]. Like phytochromes (PHYs), CBCRs utilize linear tetrapyrrole chromophores (bilins), most commonly phycocyanobilin (PCB), which undergoes reversible *Z/E* isomerization at the C15 = C16 double bond connecting the C- and D-rings, generating two distinct light-absorbing states [2]. However, while PHYs usually rely on a PAS-GAF-PHY domain architecture to enable photoconversion, CBCRs achieve full photoreversibility through the GAF (cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA) domain alone, often utilizing a single or tandemly arranged (two or more domains operating together) photoactive GAF domain which allow integration of complex light cues [3, 4]. Unlike PHYs, which primarily respond to red/far-red light, CBCRs exhibit remarkable spectral diversity, responding across the visible and near-UV range [5]. This spectral flexibility, combined with their compact size and modular architecture, underlies their well-established roles in regulating cyanobacterial processes such as phototaxis, chromatic acclimation, and circadian rhythms [5–7].

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Beyond their ecological importance, CBCRs are increasingly recognized as valuable optogenetic tools due to their broad spectral tuning and their ability to incorporate PCB when co-expressed with PCB biosynthetic enzymes in heterologous hosts such as *Escherichia coli* [8]. While a subset of CBCRs has been functionally characterized, the majority of CBCRs identified in sequenced genomes remain functionally uncharacterized, highlighting a significant gap between genomic diversity and experimental understanding [8]. Most CBCRs and PHYs carry a conserved ‘GAF Cys’ (also referred to as the first or canonical Cys) embedded in a CH or CL sequence context within the GAF domain. This residue forms the thioether linkage to the C3¹ position of the A-ring of the bilin and is the most common site of covalent attachment, although some PHYs use an N-terminal PAS Cys, and DXCIP-type CBCRs lack the GAF Cys altogether [8–10]. A subset of CBCRs additionally carries a second Cys residue within an acidic DXCF or DXCI motif (where X is variable); this second Cys forms a labile thioether to the C10 position of the chromophore in the dark-adapted state and, in many DXCF-lineage CBCRs, isomerises PCB to phycoviolobilin (PVB) by transient bond formation/breakage during the photocycle [11–13]. PVB formation is common but not universal among DXCF CBCRs; molecular determinants of efficient PVB formation have been identified and convergent PVB formation has been described in lineages employing different second-Cys positions [14–18]. Conserved residues elsewhere in the GAF pocket modulate chromophore geometry, protonation state and spectral tuning; the ‘photochromic triad’ identified in green/red CBCRs such as RcaE controls a pH-dependent, protonation-driven photocycle in which the bilin B-ring nitrogen undergoes reversible deprotonation, and is mechanistically distinct from the broader set of pocket residues (sometimes loosely termed the ‘photochromic triad’) that influences spectral tuning across CBCR families more generally [19–25]. Recent large-scale phylogenetic and mechanistic analyses provide the up-to-date framework for CBCR classification used in the present study [10, 17, 26–29]. The absorption maximum of the bilin chromophore depends on multiple factors, including π -conjugation length, chromophore geometry, protein-chromophore interactions, and protonation state. The latter can be experimentally assessed through acid denaturation assays and pH-dependent spectroscopy [11]. CBCRs are classified into subfamilies based on sequence motifs and photocycles, with recent phylogenetic classifications refining their nomenclature [6, 26, 27, 30–33]. Building on established methods for CBCR identification, systematic genome mining has proven to be a powerful approach to uncover architecturally distinct CBCR families. Such efforts not only broaden our understanding of cyanobacterial physiology but also provide new candidates

for biotechnology, as rare CBCRs offer optogenetic components with reduced crosstalk in heterologous systems [34–36].

This study recognizes a CBCR family characterized by a conserved N-terminal DUF4118 domain (Domain of Unknown Function 4118, Pfam/Interpro: PF13493), associated with membrane-associated sensor histidine kinases such as *E. coli* KdpD (GenBank ID: AAA24041.1), and a previously unrecognized DPCF-2 type GAF domain [37]. Spectral characterization of a representative member of this family, DPCF-2 CBCR (GenBank ID: QIR35741.1) from *Tolypothrix* sp. PCC 7910 revealed a blue-teal photocycle, shifting from a blue-absorbing state (Pb, $\lambda = 417$ nm) to a teal-absorbing state (Pt, $\lambda = 514$ nm) upon blue light illumination. Although blue-teal photocycles have been described previously for DXCF CBCRs from *Nostoc* spp., *T. elongatus* and *Acaryochloris marina*, the example described here extends this photocycle class to a phylogenetically distinct DPCF-2 lineage which is more closely related to DPCI-2 than to conventional DPCF. The blue-teal photocycle of this DPCF-2 CBCR occupies a spectral window distinct from the red/far-red range used by phytochrome-based optogenetic tools and the green/red range of many other CBCR-based systems. Such spectral separation may minimize the risk of unintended cross-activation when DPCF-2 CBCRs are used in conjunction with other light-responsive components within multiplexed optogenetic systems [38–40].

2 Materials and methods

2.1 General workflow and methodology

To survey architecturally distinct CBCR-TCS families within available cyanobacterial genomes, we applied a motif-guided genome mining workflow building on established approaches for CBCR identification, combining literature mining, database exploration, and computational analyses (Fig. S1) [3, 14, 30]. We initially assembled a reference dataset comprising 171 known PHYs and CBCRs by extensively reviewing published studies and genome databases, which served as the foundation for subsequent comparative analyses and identification of candidate families. To maximize the recovery of CBCRs hits, we deliberately included GAF domains from both PHYs of diverse organisms and CBCRs in the reference dataset. This decision was intended to improve the detection of distantly related or novel GAF sequences that might otherwise be missed when querying with CBCR sequences alone. To restrict the search to cyanobacterial sequences and exclude PHYs from other organisms, BLAST (Basic Local Alignment Search Tool) searches were limited to the cyanobacteria group (taxid:

1798711) in the NCBI database. Candidate selection was guided by four criteria: (1) CBCRs absent or rarely discussed in existing literature, (2) proteins containing at least one putative photoactive GAF domain, (3) those featuring atypical domains indicative of unique architecture or function, and (4) gene clusters present in significant numbers across cyanobacterial genomes.

A reference-set-based approach was preferred over a simple BLAST search because direct sequence similarity to a single query is insufficient to distinguish photoactive CBCR GAF domains from the large and diverse superfamily of non-photoactive GAF domains; the reference dataset provided the phylogenetic breadth necessary to detect distant but genuine relatives. Although the overall workflow was systematic, manual curation of BLAST outputs was an essential step: automated searches inevitably return partial open reading frames, highly redundant entries, and sequences lacking conserved chromophore-binding residues, all of which would confound downstream phylogenetic and domain analyses. Critically, motif-level analysis using MEME (Multiple Em for Motif Elicitation), applied specifically to the GAF domain region, allowed us to identify the conserved DXCF/DXCI sequence patterns and dual-cysteine configurations that are the hallmarks of photoactive CBCRs, thereby providing a sequence-based filter that distinguishes candidate photoreceptors from the broader pool of GAF-containing proteins with non-photosensory functions. This systematic screening strategy enabled the prioritization of CBCRs with distinctive structural and regulatory features not seen in previously characterized families. Among the candidate sequences, we observed a notable predominance of GAF domain-containing proteins associated with the conserved N-terminal DUF4118 domain (37.8% of the total), which represents a tetrahelical module of yet unknown function [37]. To validate their potential as photoactive CBCRs, we conducted sequence analyses to confirm the presence of conserved chromophore-binding residues within the GAF domains [8, 14, 33, 41, 42]. Genomic context analysis further revealed co-occurrence of response regulators, transcriptional elements, and signaling components, strongly supporting their integration into TCS pathways.

2.2 Sequence alignment and phylogenetic tree

The workflow proceeded in two complementary stages, summarised in Fig. S1. Stage 1 was designed to maximise the architectural diversity of the recovered GAF-domain proteins, and Stage 2 was a focused expansion aimed specifically at recovering additional DUF4118-containing CBCRs that the diversity-driven Stage 1 might not have captured. In Stage 1, we began with a reference set of 171

known PHYs and CBCRs compiled from published studies and genome databases. From this set, 48 GAF-domain sequences were chosen to span the broadest possible architectural and phylogenetic diversity and used as queries for PSI-BLAST against cyanobacterial genomes (taxid: 1798711) in the GenBank database [43]. To identify conserved motif patterns across the recovered sequences, we used the MEME suite version 5.5.3 (Fig. S2) [42]. Individual targets were frequently recovered by multiple queries, with the median target hit by approximately six independent queries; this redundancy across the diverse query set served as an internal consistency check that the search had reached saturation. After manual curation to remove partial open reading frames, collapse redundant entries returned by multiple queries, deduplicate sequences with > 95% pairwise identity, and confirm the presence of a recognisable GAF domain by Pfam annotation, we obtained a refined set of 2,297 unique sequences from approximately 1,900 cyanobacterial genomes. From this pool, 82 candidates with atypical or rare domain architectures were carried forward for closer inspection (Table S1). Of these 82, 31 contained a conserved N-terminal DUF4118 domain (Fig. S4), making DUF4118 the most prevalent unusual domain in this set. Because Stage 1 was driven by a diverse set of GAF queries rather than by DUF4118 itself, we conducted a focused Stage 2 search using the 31 DUF4118-containing hits as queries to recover additional DUF4118 + GAF proteins that Stage 1 might not have captured. This yielded a further 90 DUF4118 + GAF sequences, for a combined non-redundant dataset of 121 DUF4118 + GAF proteins. After removal of sequences lacking a recognisable photoactive GAF, 100 sequences were retained for downstream multiple sequence alignment, phylogenetic tree construction, and synteny analysis (Fig. S5). The sequences were aligned with the MUSCLE algorithm in Jalview v2.11, and phylogenetic reconstruction was performed using the PAML method with 1,000 bootstrap replicates in MEGA X [44, 45]. Phylogenetic relationships were integrated with domain architecture analysis using TBtools v2.0, with bulk domain assignments retrieved through the NCBI Batch CD-Search Tool against the Pfam database [46, 47].

Many non-CBCR signalling proteins in cyanobacteria also possess GAF domains, so distinguishing putative CBCRs from non-photosensory GAF proteins requires identification of conserved photoactive motifs and residues. The GAF sequences of the 100 DUF4118-associated proteins were therefore subjected to multiple sequence analysis to identify characteristic motifs and residues indicative of photoactivity (Fig. S6). The criteria below were designed to identify dual-Cys, DXCF/DXCI-type CBCRs in previously unrecognised architectural contexts; they are not intended as a universal CBCR classifier

and intentionally exclude well-characterised single-Cys CBCRs such as RcaE and Slr1393g3. For the purposes of this study, candidate sequences were retained when all three of the following features co-occurred within a GAF domain: (1) a conserved GAF Cys (first Cys, in a CH or CL context) at the canonical A-ring attachment position; (2) a second Cys embedded in an acidic DXCF or DXCI motif (corresponding to C522 in *TePixJ*); and (3) a recognisable acidic DPCF, DPCI or related variant N-terminal to the second Cys. Sequences meeting all three criteria were considered candidate photoactive DXCF-type CBCRs for the purposes of downstream phylogenetic and architectural analysis. Of the 100 sequences examined, 10 DUF4118-CBCR loci, comprising 5 DPCI-2 and 5 DPCF-2 subgroup members, were carried forward for detailed synteny analysis alongside reference DPCI and DPCF GAF sequences from the literature. The DPCF-2 CBCR from *Tolypothrix* sp. PCC 7910 (QIR35741.1) was selected from this set for heterologous expression and spectral characterisation, as described in Sect. 3.4.

2.3 Synteny analysis

The genomic neighborhoods of all DUF4118 domain-containing CBCRs (100 sequences) were analyzed and visualized using Gene Graphics, a genomic neighborhood data visualization web application (Fig. 1) [48]. This allowed for the comparative analysis of gene arrangements and potential functional associations within these regions. To ensure accuracy, incomplete outputs were manually verified and supplemented using the graphics module of NCBI Nuccore (nucleotide database), enabling precise annotation and validation of genomic contexts. To further characterize the structural composition of these genomic regions, the domain architecture of individual components was retrieved and analyzed. Functional domain patterns were identified using the NCBI Batch CD-Search Tool (leveraging the Pfam domain database) for precise annotation, while TBtools was used to organize and visually present the domain arrangements (Fig. S7) [46, 47].

2.4 Expression and purification of DPCF-2 CBCR GAF domain

The genetic sequence encoding the DPCF-2 GAF domain (residues 248–422 of QIR35741.1) from *Tolypothrix* sp. PCC 7910 was codon-optimized for expression in *E. coli* (Genscript) to improve translational efficiency, given the AT-rich codon usage typical of cyanobacterial genomes. The optimized sequence was synthesized and cloned into the pET21b(+) expression vector (Genscript), which

appends a C-terminal His₆-tag used for downstream Ni-NTA affinity purification. For protein expression, *E. coli* BL21(DE3) cells were cultivated in Terrific Broth (TB) medium at 37 °C with shaking at 200 rpm. When the culture reached an optical density (OD₆₀₀) of 0.5, protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was further incubated for 16 h at 22 °C. Bacterial cells were harvested and resuspended in buffer A (50 mM sodium phosphate, 150 mM NaCl, pH 8.0) and lysed with an EmulsiFlex® high-pressure homogenizer. For PCB chromophore incorporation, co-expression of PCB biosynthetic enzymes within the same culture was attempted but yielded insufficient chromophore loading under the conditions tested. PCB was therefore provided by mixing the soluble fraction of the DPCF-2 GAF domain-expressing culture with a cleared lysate from a separate *E. coli* BL21(DE3) culture co-expressing the heme oxygenase HO1 (Slr1184, GenBank ID: BAA16864.1) and the ferredoxin-dependent bilin reductase PcyA (Slr0116, GenBank ID: BAA10653.1) of *Synechocystis* sp. PCC 6803, cloned into a pCDFDuet-1 backbone [49, 50]. The mixed lysates were incubated overnight at room temperature without addition of exogenous reducing agents; the procedure relied on PCB produced during prior in-vivo co-expression of HO1 and PcyA in the host *E. coli*. The resulting holoprotein was purified using Ni-NTA affinity chromatography in buffer A with a 0–150 mM imidazole gradient. To eliminate residual imidazole, which can interfere with chromophore spectroscopy, the buffer was exchanged back to buffer A by multiple consecutive dilution and concentration rounds using Amicon Ultra centrifugal filters (10 kDa MWCO, Millipore). The protein was then concentrated to 5–15 mg/ml, flash-frozen in liquid nitrogen, and stored at – 80 °C. See Fig. S9 for SDS-PAGE purity assessment, zinc induced fluorescence detection for bilin-binding proteins, and absorbance spectra of the purified holoprotein [51].

2.5 UV-Vis spectroscopy of DPCF-2 CBCR GAF domain

The UV-Vis measurements were executed with an Agilent Cary 8454 UV-Visible spectrophotometer (Agilent) at 23 °C. For the dark and illuminated state spectra and dark reversion measurements, the samples were diluted in buffer (50 mM sodium phosphate, 150 mM NaCl, pH 8.0) to obtain an absorbance of approximately 0.3 at the bilin-specific absorption peaks before data normalization. The samples were illuminated in all experiments with saturating blue (463 nm; 160 μW/cm² or ~ 6.2 μmol m⁻² s⁻¹) or green (518 nm; 100 μW/cm² or ~ 4.3 μmol

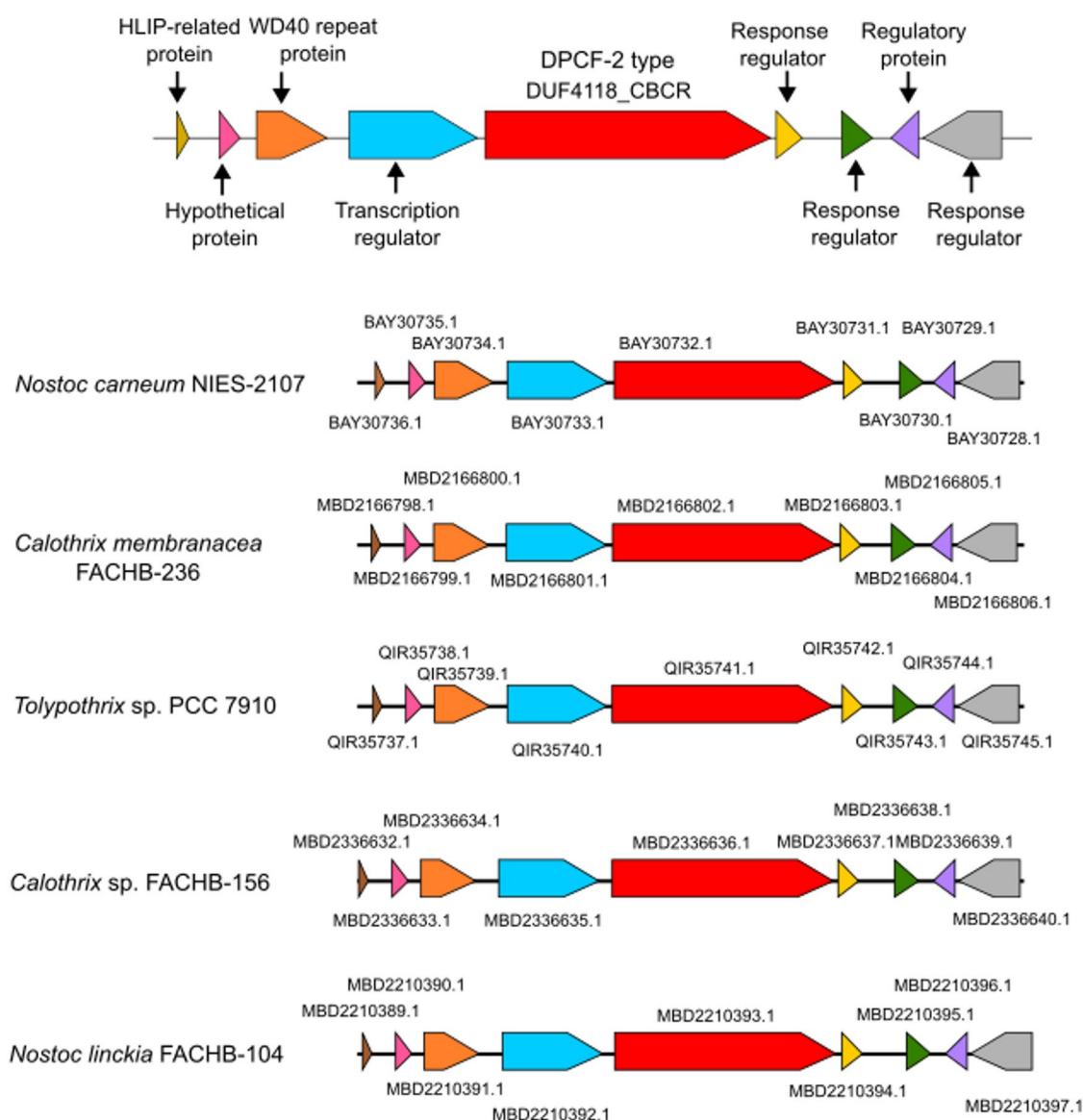


Fig. 1 Genomic organization of DPCF-2 type CBCR gene clusters across various cyanobacterial species. The DPCF-2-type CBCR is depicted in red, flanked by a variety of regulatory and functional elements. These include hypothetical proteins (pink), HLIP-related proteins (brown), WD40-repeat proteins (orange), transcriptional regulators (blue), response regulators (yellow and green), and additional regulatory proteins (purple and gray). Each gene is annotated with its corresponding GenBank accession number. Arrow length indicates

relative gene size. The gene clusters are drawn to scale and illustrate a conserved modular organization across five filamentous cyanobacterial species: *Nostoc carneum* NIES-2107, *Calothrix membranacea* FACHB-236, *Tolypothrix* sp. PCC 7910, *Calothrix* sp. FACHB-156, and *Nostoc linckia* FACHB-104. The conservation of gene synteny and overall architecture suggests a shared regulatory logic and potential functional convergence of these CBCR-linked TCS systems in response to specific light cues

$\text{m}^{-2} \text{s}^{-1}$) light-emitting diodes. In all cases, sample was illuminated until photoequilibrium was reached (up to 10 min for blue and 20 min for green light). See Fig. S10i for emission spectra of the light sources. For urea denaturation, sample was either kept in dark or illuminated with blue LED to the photoequilibrium and mixed with 10 M urea pH 3 to yield final urea concentration of 8 M [30, 52, 53]. Data acquisition of the denatured sample was then conducted in dark or under subsequent blue/green

illumination. All absorption spectra were baseline corrected at 850 nm and normalized to A_{280} .

2.6 Structure prediction of the DPCF-2 type DUF4118_CBCR from *Tolypothrix* sp. PCC 7910

The full-length amino acid sequence of the DPCF-2 type DUF4118_CBCR from *Tolypothrix* sp. PCC 7910 was retrieved from the NCBI database (GenBank ID:

QIR35741.1). Domain boundaries were identified using InterProScan and Pfam annotations to guide modeling of functional regions, including the DUF4118, GAF, PAS, HisKA, and HATPase domains. Protein structure prediction was performed using AlphaFold3 [54]. Given the large size of the protein, predictions were generated in two fragments corresponding to N-terminal and C-terminal regions, with the domain boundary indicated in Fig. 4A. The protein was modeled as a homodimer, consistent with known architectures of histidine kinase-associated photoreceptors. Structural models were visualized, analyzed, and assembled in PyMOL v2.5 (Schrodinger, LLC). To assess the accuracy of the predicted domains, the DUF4118 region was compared to the NMR solution structure of the *E. coli* KdpD sensory domain (PDB ID: 2ksf) using PyMOL structural alignment tools. The GAF domain was aligned to the crystal structure of TePixJ (PDB ID: 4glq). Two blue-state crystal structures of TePixJ are available from the same study [31]. GLQ (1.6 Å resolution) and 4FOF (2.0 Å resolution). We selected 4GLQ as the primary reference on the basis of its higher resolution. The principal structural difference between the two is the orientation of the bilin A-ring, while the chromophore-binding pocket residues, including the DPCF motif and the two chromophore-binding cysteines (C494 and C522, corresponding to C326 and C354 in the *Tolypothrix* DPCF-2 CBCR), are conserved in both structures. The conclusions and interpretation drawn here would therefore be unchanged using 4FOF as the reference. Root mean square deviation (RMSD) values were calculated using the align command in PyMOL. Conserved motifs and cysteine residues implicated in chromophore attachment were identified by multiple sequence alignment of the *Tolypothrix* DUF4118_CBCR with TePixJ. The DPCF motif (residues 492–495) and chromophore-binding cysteines (C326 and C354 in *Tolypothrix*; corresponding to C494 and C522 in TePixJ) were inspected in the structural model. Chromophore arrangement was inferred by comparison with the bound PVB structure in TePixJ (PDB ID: 4glq).

3 Results

3.1 DUF4118 domain-containing CBCRs exhibit a diverse range of domain architectures

Following the workflow for classifying novel CBCR-TCS families in cyanobacterial genomes (Fig. S1), we first catalogued the domain organization of all 171 reference PHYs and CBCRs in our dataset (Fig. S2). This step was necessary to define the domain architecture space already covered by known proteins, so that proteins with genuinely novel or rare domain combinations could be systematically

distinguished from previously recognized families. From this reference set, we selected 48 GAF domain sequences that together provided the broadest possible coverage of known sequence diversity, maximizing the chance that PSI-BLAST searches would recover the full range of GAF-domain-containing proteins across cyanobacterial genomes. The GAF domains within these proteins exhibited a broad spectrum of motif patterns (Fig. S3). The outcome of our analysis revealed proteins with unique domains, with noteworthy instances being: DUF4118 (31 hits), CHASE3 (20 hits), DUF745 (10 hits), MASE1 (7 hits), Prominin (6 hits), CHASE (5 hits), and AA_permease_2 (3 hits) (Table S1). Among these results, proteins containing DUF4118 domain were present in the largest number (Fig. S4). The DUF4118 proteins exhibited domain arrangements typical to GAF domain-containing sensor histidine kinases and CBCRs (Figs. S4 and S5). In all instances, DUF4118 domains were present at N-terminus and some of the proteins also contained a second GAF domain. We used these proteins as ‘query’ to further identify similar proteins in the cyanobacterial genomes available in the NCBI GenBank database. A total of 90 proteins were identified that contained both DUF4118 and GAF domain (Fig. S5). These proteins are distributed across three distinct orders of cyanobacteria: Oscillatoriales, Nostocales, and Chroococcales. Within the Oscillatoriales order, the DUF4118_CBCR subgroup exhibited some members featuring an additional photoresponsive DTYL-type (Asp-Thr-Tyr-Leu) GAF domain belonging to XRG (eXpanded Red-Green) lineage, suggesting a potentially more intricate spectral sensing mechanism within this subset [13]. Within the Nostocales order, DUF4118_CBCRs with a single GAF domain constituted the largest subgroup in terms of sequence numbers among the three cyanobacterial orders examined.

3.2 DUF4118_CBCRs contain DPCI, DPCF and DTYL type GAF domains

The multiple sequence alignment of GAF domains demonstrates that the majority of first GAF (g1) domains belong to the DPCX (DPCI and DPCF) type (Fig. S5) containing the highly conserved photochromic triad Leu-Thr-Lys (L-T-K) (Fig. S6). The majority of first GAF (g1) domains contain DPCX-type motifs, where X is a variable residue (I = isoleucine and F = phenylalanine) and C is the secondary cysteine involved in bilin isomerization (Fig. S5) [19, 35–37, 41, 55]. Interestingly, DPCF motifs in DUF4118_CBCR are more closely related to DPCI-2 GAFs than to conventional DPCF GAFs (Fig. 2). Such DPCF type GAFs have not been described before and due to their closeness to DPCI-2 family we named them DPCF-2 type in this study (Fig. S6). The second GAF [g2] domains in some DUF4118_CBCR

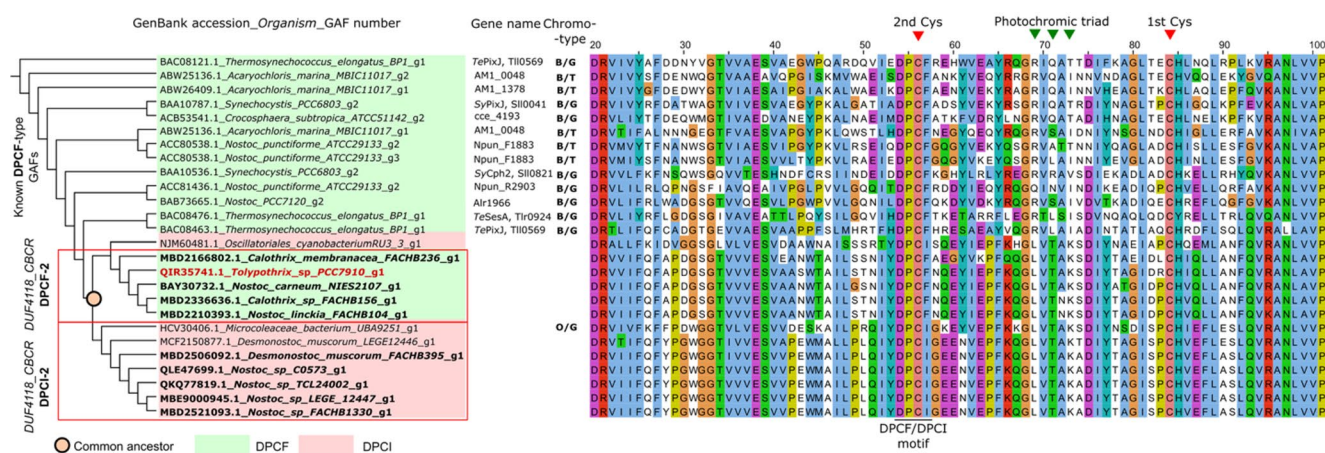


Fig. 2 Phylogenetic tree and sequence alignment of DPCF-2 and DPCI-2 type CBCRs within the DUF4118_CBCR family. The left panel shows a phylogenetic tree, with DPCF-type sequences highlighted in green and DPCI-type sequences in red. A common ancestor is indicated by a beige circle. The right panel presents a multiple sequence alignment of key residues within the DPCF/DPCI motif, with color-coded amino acids. The chromotypes for each sequence were assigned based on the spectral properties reported in the literature

are primarily of the DTYL type of XRG lineage (Fig. S5) [56]. A distinctive subset of DUF4118-containing proteins, exclusive to the Chroococcales order, lacks the conserved GAF Cys and other chromophore-coordinating residues, making it difficult to classify them as photochemically active CBCRs on sequence grounds alone. Whether these GAF domains nevertheless retain a viable bilin-binding pocket, for example, by employing an alternative covalent attachment site or by binding chromophore non-covalently, is a question to be addressed in the future with a combination of modelling and biochemical reconstitution.

3.3 Two sister families of DUF4118_CBCRs contain conserved TCS systems

We identified two closely related DUF4118_CBCR subfamilies, one containing a PAS domain followed by a GAF and five PAS domains and other containing a GAF domain followed by two PAS domains (Fig. S7). The latter one contains a DPCI-2 type GAF as described by Hoshino & Narikawa (2022) and the former one contains the DPCF-2 type GAF [30, 31, 41, 42]. Immediately flanking the DPCF-2 type CBCR locus are open reading frames predicted to encode a WD40-repeat protein, a winged-helix transcriptional regulator, and one or more response regulators belonging to TCS systems (Fig. 1).

Additional neighboring genes encode hypothetical proteins, high-light-inducible (HLIP-related) proteins, and auxiliary sensory or enzymatic components [57–59]. Similarly, in the genomic neighborhood of DPCI-2 type CBCRs, the genes encoding a WD40-repeat protein, a winged-helix

transcriptional regulator, a response regulator, three hypothetical proteins, a pyruvate: ferredoxin oxidoreductase, a sulfurtransferase, and a DUF1499 domain-containing protein are present. This conserved clustering pattern suggests that DPCI-2 type CBCRs are also embedded within complex regulatory modules that integrate redox, metabolic, and light-dependent signals (Fig. 2).

3.4 DPCF-2 CBCR from *Tolypothrix* sp. PCC 7910 exhibits a blue-teal photocycle

To study the spectral properties of this architecturally distinct CBCR family, we characterized the GAF domain of the DPCF-2 CBCR (QIR35741.1) from *Tolypothrix* sp. PCC 7910 by UV-Vis absorption spectroscopy (Figs. 3 and S10). This protein was selected as a representative family member because it is an individually deposited GenBank entry from a publicly available Pasteur Culture Collection strain and sits at the centre of a tightly conserved Nostocales clade (Fig. 2). We note at the outset that the purified preparation contained substantial co-eluting protein impurities (Fig. S9), as reflected in the relatively high A280 compared to the bilin-specific bands. As discussed below, the diagnostic photochemical signatures and the acid-urea denaturation products demonstrate that the spectral features attributed to a bilin chromophore arise from the target DPCF-2 GAF domain rather than from co-purifying contaminants. In the dark-adapted state, the protein exhibited a primary absorption peak at 417 nm that falls within the blue region (410–480 nm) whereas exposure to blue light produced a 514-nm

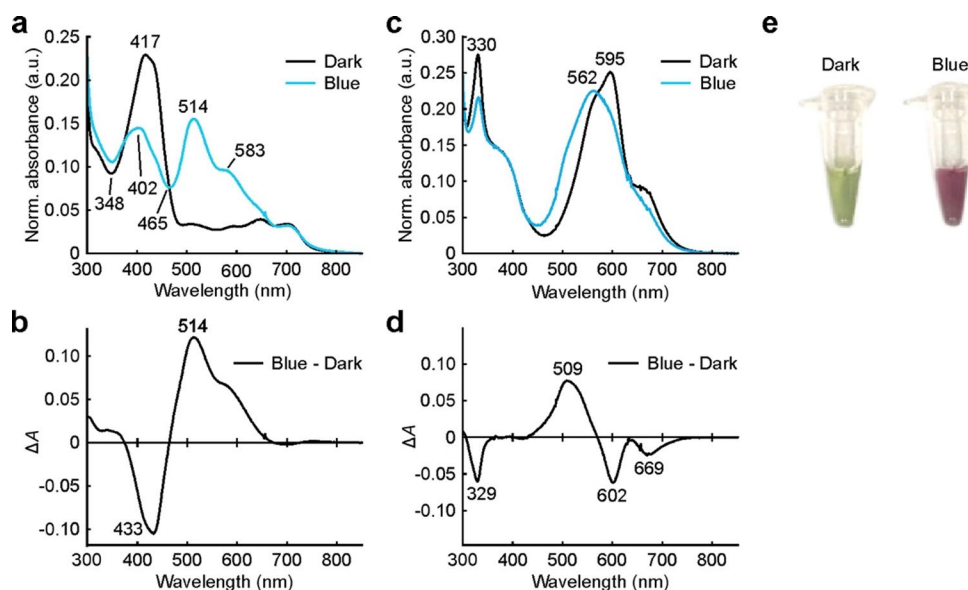


Fig. 3 Absorbance spectra of the GAF Domain in DPCF-2 CBCR from *Tolypothrix* sp. PCC 7910. **a** Absorbance spectra and **b** a difference spectrum of the protein in dark and after saturating blue illumination (463 nm, $\sim 6.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, 10 min). The spectra show a peak shift from 417 nm to 514 nm upon illumination, indicative of a blue-teal photocycle. **c** Absorbance spectra and **d** a difference spectrum of dark-adapted or blue-pre-illuminated (463 nm, $\sim 6.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, 10 min) samples after acidic urea denaturation (8 M urea, pH 3). The 595 nm

and 562 nm peaks correspond to 15Z-PVB and 15E-PVB, respectively, therefore supporting Z/E isomerization upon photoconversion [52]. **e** The color change of the protein solution from green to reddish-violet hue by blue light. For more extensive spectral characterization, see Fig. S10. The color classifications in this study are defined as follows: near-UV (300–395 nm), violet (395–410 nm), blue (410–480 nm), teal (480–520 nm), green (520–570 nm), yellow (570–580 nm), orange (580–615 nm), red (615–685 nm), and far-red (685–750 nm) [10]

peak at the teal region (480–520 nm) [30] (Fig. 3a). This transition is consistent with a GAF photoconversion mechanism between a dark-adapted blue-absorbing (Pb) state and a blue illuminated teal-absorbing (Pt) state [60, 61], which was also evident as a color shift from green to a reddish-violet hue of the sample (Fig. 3e). The photoconversion was fully reversible, as the resting Pb-state spectrum could be reproduced by illuminating the Pt state with green light (Fig. S10a–c). We did not detect notable dark reversion from Pt state at room temperature (Fig. S10g), as also reported for GAF domain from *Thermosynechococcus elongatus* PixJ (*TePixJ*) [11, 62]. Interestingly, blue and green light illumination produced an additional absorption peak at 583 nm (Fig. 3a, Fig. S10a–c), which required extensive illumination lengths to disappear (Fig. S10h). This additional peak may be the result of a breakage of the second thioether linkage and thus formation of PCB from PVB upon photoconversion, as also proposed for a 580-nm peak that appeared for *TePixJ* GAF domain upon Pg-to-Pb photoconversion [63, 64].

Urea denaturation of the dark-adapted Pb-state sample produced a major absorption peak at 595 nm, while the blue-illuminated Pt-state denatured sample peaked at 562 nm (Figs. 3c and S10d, e). These peak positions are consistent with the 15Z- and 15E-isomers of PVB respectively, confirming Z/E isomerisation upon photoconversion [14, 30, 41]. In addition to 595-nm peak, the dark-adapted

Pb-state spectrum had a long-wavelength shoulder at ~ 670 nm indicative of PCB species, which is also supported by a 669 nm peak in the difference spectrum (Fig. 3d). Based on the Pb-state denatured spectrum and the known extinction coefficients of PVB (ϵ_{590} 38 600) and PCB at 662 nm (ϵ_{662} 35 500), and assuming minimal overlap between the PVB- and PCB-specific peaks, we estimated the PVB:PCB ratio being roughly 2.4 [65]. This indicates that the holoprotein binds a mixed chromophore population in which PVB is the majority species and PCB the minority. The denatured Pt-state was readily reverted to the Pb-state by green illumination, whereas the denatured Pb-state was not converted by blue illumination (Fig. S10d, e); this asymmetry is the expected photochemistry for denatured PVB, in which efficient Z-to-E isomerisation requires a protein environment [66]. The similar shapes of the native (Fig. S10c) and denatured Pt difference spectra (Fig. S10f) indicate that the E-to-Z isomerisation of the denatured Pt form proceeds along the same trajectory as in the native protein.

Four independent lines of evidence together support the assignment of the bilin-region spectral features to the DPCF-2 GAF holoprotein rather than to a co-purifying contaminant: (i) zinc-induced fluorescence detection on the SDS-PAGE gel (Fig. S9a) labels a single bilin-binding species at the expected molecular mass of the recombinant DPCF-2 GAF construct (~ 21.8 kDa), with no fluorescent signal in any of the contaminating Coomassie-stained bands;

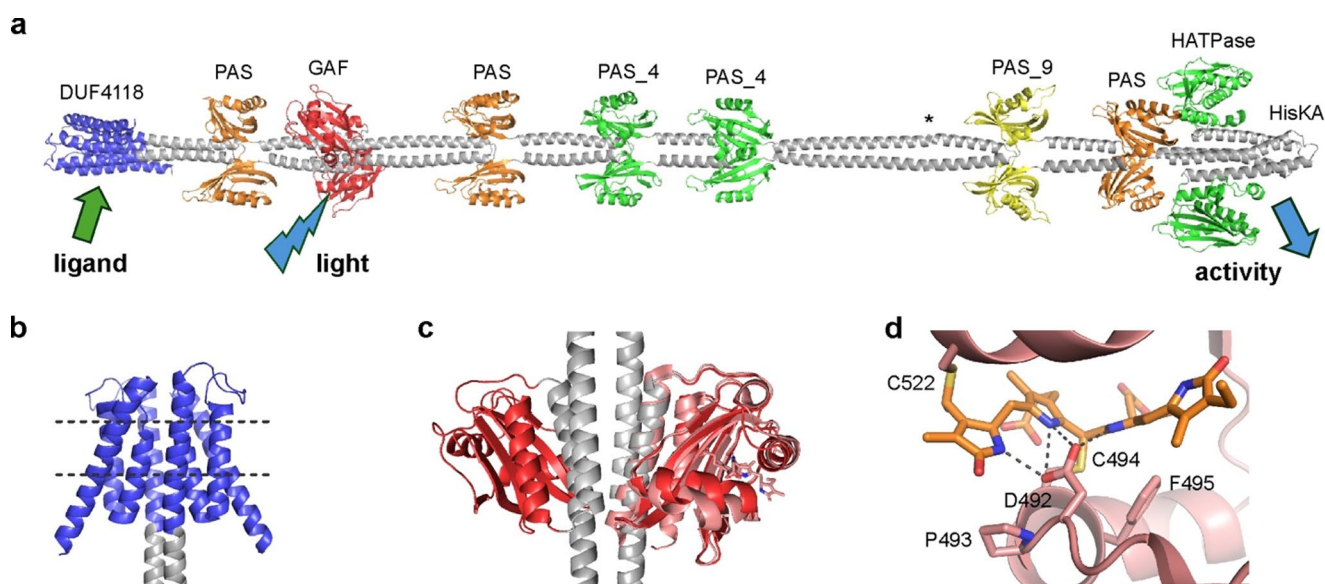


Fig. 4 Structure of a DPCF-2 type CBCR with a DUF4118 domain. **a** AlphaFold3 prediction of full-length DPCF-2 type DUF4118_CBCR from *Tolypothrix* sp. PCC 7910. The prediction of this 153 kDa protein (1344 residues, Fig. S8b) was generated assuming a dimeric arrangement and in two fragments, the boundary of which is indicated as an asterisk (*). **(b)** Closer view of the DUF4118 domain shows a four-helical structure, which agrees with the solution structure of *E. coli* KdpD [37]. **(c)** Model of the GAF region (red) aligned with the crys-

tal structure of a GAF domain from *TePixJ* (pink, PDB ID: 4glq). **(d)** Structure of PVB chromophore and associated ⁴⁹²DPCF⁴⁹⁵ motif in *TePixJ* (PDB ID: 4glq). Based on the conservation of the two chromophore associated cysteines (C494 and C522) also in DPCF-2 type DUF4118_CBCR (C326 and C354), the PCB likely forms two thioether linkages and adopts PVB-like arrangement. Structural figures were generated with PyMOL v2.5 software (Schrödinger, LLC)

(ii) the absorption peaks at 417 nm and 514 nm fall in the bilin region (> 350 nm), where *E. coli* house-keeping proteins do not absorb; (iii) the photoconversion between these two states is fully reversible under blue/green illumination, a hallmark of bilin-bound CBCR GAF domains and not of any colored *E. coli* contaminant; and (iv) acid-urea denaturation released chromophore species absorbing at 595 nm and 562 nm, the canonical 15Z- and 15E-PVB peaks, with the denatured chromophore exhibiting the asymmetric photochemistry previously reported for PVB in *TePixJ* [63].

3.5 Structural features of DPCF-2 type DUF4118_CBCR from *Tolypothrix* sp. PCC 7910

The full-length DUF4118_CBCR from *Tolypothrix* sp. PCC 7910 was modeled as a homodimer using AlphaFold3 [54] (Fig. 4a). The model exhibited a modular organization comprising an N-terminal DUF4118 domain, a light-responsive GAF domain, a series of PAS domains, and a C-terminal HisKA-HATPase catalytic module. Within the N-terminal region, the DUF4118 domain adopted a compact four-helix bundle (Fig. 4b), resembling the sensory domain of *E. coli* KdpD, suggesting a function in environmental signal detection [37]. Inspection of the DUF4118 domain model did not reveal a clearly defined ligand-binding cleft or spatially conserved residue cluster analogous to those seen

in experimentally characterized ligand-sensing domains. While the overall fold is consistent with signal-sensing four-helical bundles, the structural basis for any putative ligand interaction remains to be established through experimental approaches such as co-crystallography or hydrogen-deuterium exchange mass spectrometry [67]. The GAF domain responsible for chromophore binding showed strong structural correspondence to the CBCR *TePixJ* (GenBank ID: BAC08121.1; T110569; PDB: 4glq), with a root-mean-square deviation of 0.870 Å across 122 of 144 aligned residues (Fig. 4c). Analysis of the chromophore-binding pocket revealed that the key DPCF motif (residues 492–495) and the two cysteines required for bilin attachment (C326 and C354) are retained (Fig. 2). Residue numbering hereafter is given as '*Tolypothrix*-DPCF-2 / *TePixJ*', with the first index referring to the *Tolypothrix* sp. PCC 7910 DPCF-2 CBCR (QIR35741.1) and the second to *TePixJ* (T110569). The two chromophore-binding cysteines C326 / C354 in the *Tolypothrix* DPCF-2 CBCR correspond to C494 / C522 in *TePixJ* (Fig. 4d), and together with the spectral characterisation (Fig. 3, Fig. S10) support the formation of dual thioether linkages to the PCB chromophore and a PVB-like arrangement. Taken together, these structural features suggest that DPCF-2 type CBCRs operate as integrated sensory systems, coupling ligand and light cues to downstream histidine kinase signaling through their modular, multi-domain arrangement.

4 Discussion

This study presents the discovery and spectral characterization of a newly classified CBCR featuring a conserved DUF4118 domain and a previously unrecognized DPCF-2 type GAF domain. DUF4118 is structurally related to the sensory domain of KdpD, a sensor histidine kinase that responds to osmotic stress, suggesting that DUF4118-containing CBCRs may integrate ligand or environmental cues alongside light perception. However, this remains a structural inference as no binding cleft was identified in the predicted model, and experimental validation of ligand binding will be required to substantiate this hypothesis [37]. Nevertheless, CBCRs containing both a DUF4118 and a GAF domain may function as dual-input signaling proteins, integrating light perception and chemical/ligand sensing within a single regulatory system. Such integration may enable cyanobacteria to fine-tune their responses to changing environmental conditions, likely optimizing processes such as photosynthesis, motility, and metabolic adaptation [8, 68, 69]. Since DUF4118_CBCRs are found only in a small number of species, they likely play a specialized regulatory role rather than being a universally required photoreceptor in cyanobacteria (Figs. 1, 2 and S5).

Many members of the Nostocales are heterocyst-forming, nitrogen-fixing cyanobacteria such as *Nostoc* and *Tolypothrix* that thrive in diverse but specific ecological niches, including freshwater habitats, biological soil crusts, and symbiotic associations [70–73]. The restricted taxonomic distribution of DUF4118_CBCRs, predominantly within the Nostocales, raises the possibility that they participate in lineage-specific regulatory processes. Plausible candidates include the regulation of heterocyst differentiation, light-modulated control of N₂ fixation, photoprotective acclimation, biofilm or akinete formation, and modulation of filamentous growth or motility. We note, however, that filamentous morphology itself is not unique to the Nostocales but is also widespread among Oscillatoriales and other orders, and that nitrogen fixation per se is broadly distributed across cyanobacteria, including unicellular taxa (*Crocospaera*, *Cyanothece*, the UCYN-A symbiont) and non-heterocystous filamentous taxa. Our argument therefore concerns the heterocyst-forming biology of Nostocales specifically, rather than filamentation or nitrogen fixation as broader traits. Because the conserved TCS architecture and the blue-teal photocycle are equally compatible with any of these candidate roles, in vivo genetic dissection, for example co-expression studies, pull-down assays, promoter activity measurements, and targeted mutagenesis in heterocystous Nostocales strains, will be required to discriminate among them.

The identification of two major subfamilies of DUF4118-containing CBCRs, one featuring a DPCI-2-type GAF domain and the other with the newly described DPCF-2-type GAF domain, provides valuable insights into the evolutionary divergence of these photoreceptors (Fig. 2). Phylogenetic analysis and sequence comparisons indicate that DPCF-2-type CBCRs are evolutionarily closer to DPCI-2 type CBCRs than to other DPCF CBCR lineages [30, 31, 42]. This close relationship suggests that DPCF-2 CBCRs likely emerged through speciation followed by functional divergence, enabling them to optimize their light-sensing properties for specific environmental conditions. The conservation between the DPCF-2 and DPCI-2 GAF types, including a Leu-Thr-Lys (L-T-K) sequence motif at positions equivalent to the triad described in other DXCF CBCRs (Fig. 2, S6), suggests that DPCF-2 CBCRs retain residues likely to influence chromophore geometry, protonation state and photoconversion efficiency [25, 30]. We emphasise, however, that this assignment is based on sequence conservation and positional correspondence alone; site-directed mutagenesis will be required to test whether these residues actually contribute to the DPCF-2 photocycle [22]. We use the term ‘photochromic triad’ here in the broad, sequence-defined sense, which should not be conflated with the mechanistically distinct ‘protochromic triad’ of green/red CBCRs such as RcaE [19], which controls a pH-driven photocycle through chromophore pK_a modulation [19–21]. Phylogenetic clustering further supports this relationship, as DPCF-2 CBCRs form a distinct but closely related clade to DPCI-2 CBCRs. The emergence of DPCF-2 type CBCRs through duplication and divergence allowed for functional specialization, particularly in spectral tuning, which may provide adaptive advantages in specific ecological niches.

The spectral characterization of the DPCF-2 GAF domain from *Tolypothrix* sp. PCC 7910 revealed a photochromic transition between a blue-absorbing state (Pb, 417 nm) and a teal-absorbing state (Pt, 514 nm) (Fig. 3). This transition is fully reversible and involves Z-to-E isomerization of PVB chromophore (Figs. 3 and S10). This blue-teal photocycle is distinct from the dominant CBCR photocycles characterized to date, like blue/green, red/green and green/red, as well as from the red/far-red photocycle of canonical PHYs [50, 61]. Teal-absorbing photoproducts are nonetheless not unprecedented: they have been reported in blue/teal CBCRs such as SesB [60, 74] and in green/teal CBCRs such as DpxA [60, 75]. Functionally, blue-sensing CBCRs are not necessarily restricted to direct light-quality detection: blue/green CBCRs have been shown to function as shade sensors that respond to the spectral changes characteristic of canopy or self-shading conditions [60, 74]. By analogy, the teal-absorbing photoproduct of the DPCF-2 CBCR could contribute a secondary ‘tuning’ or modulatory output,

analogous to the role of DpxA in fine-tuning the principal regulator alongside RcaE in complementary chromatic acclimation [60, 75]. Blue-teal photocycles have been previously reported in other CBCRs, including Tlr1999/SesB (GenBank ID: BAC09551.1) from *T. elongatus*, NpF1883g2/3/4 from *Nostoc punctiforme* (GenBank ID: ACC80538.1), and AM1_0048g2 from *Acaryochloris marina* (GenBank ID: ABW25136.1), establishing this spectral range as functionally relevant in cyanobacterial photobiology [30, 74, 76, 77]. The DPCF-2 CBCRs described here extend this class to a phylogenetically distinct lineage, one in which blue-teal sensing occurs within the unique architectural context of a DUF4118-containing, multi-domain photoreceptor consistently linked to TCS systems. This combination of spectral behavior and domain architecture has not been previously described and may reflect an evolutionary adaptation enabling the integration of light and ligand cues within a single regulatory protein. However, as only one representative member has been spectroscopically characterized to date, broader conclusions about the photochemical and functional properties of this family await characterization of additional members. The distinct blue-to-teal photocycle suggests that DPCF-2 CBCRs have adapted to specific environmental lighting conditions, particularly in low-light aquatic ecosystems, where spectral availability is significantly different from terrestrial habitats. Blue light is among the most penetrating wavelengths in water, whereas red and far-red light are quickly absorbed by water and photosynthetic pigments. This adaptation implies that DPCF-2 CBCRs may function in environments where blue light is more prevalent, such as deep-water layers, shaded microbial mats, or turbid aquatic ecosystems. The blue-to-teal spectral range (417–514 nm) is notably well-matched to the light available in depth-stratified aquatic environments, where longer wavelengths are rapidly attenuated. While the downstream cellular targets of DPCF-2 CBCR signaling remain unknown, the conserved TCS architecture, comprising a histidine kinase coupled to multiple downstream response regulators, provides a defined molecular framework for future functional dissection in cyanobacterial expression systems [4, 69, 76, 78].

The genomic context of DUF4118_CBCR genes provides evidence for their integration into conserved cyanobacterial signaling modules. Critically, a nearly identical gene cluster organization, CBCR flanked by a WD40-repeat protein, a winged-helix transcriptional regulator, and multiple response regulators, is preserved across five phylogenetically distinct Nostocales species (Figs. 1 and S7). This cross-species conservation of gene synteny, rather than genomic context in any single organism alone, substantially increases the probability that these CBCR loci participate in a shared and functionally coherent regulatory pathway. WD40-repeat proteins, though rare in most bacteria, are

notably enriched in cyanobacteria, where they are implicated in protein-protein interaction scaffolding within regulatory complexes; their recurrent co-occurrence with DUF4118_CBCRs across multiple genomes is therefore consistent with a conserved, multi-protein signaling module responsive to light input [57, 58].

The full-length DUF4118_CBCR architecture, combining a putative ligand-sensing DUF4118 domain with a light-responsive GAF domain within a single histidine kinase-linked protein, suggests a dual-input signaling paradigm in which both light and chemical/environmental cues could be integrated into a unified regulatory output. This architecture is further supported by in silico modeling (Fig. 4a-c), which shows the structural plausibility of this functional bifurcation between light and ligand perception. AlphaFold3-predicted structures of the full-length DUF4118_CBCR from *Tolypothrix* sp. PCC 7910 suggest a modular arrangement, with the DUF4118 domain adopting a four-helical bundle configuration reminiscent of known ligand-sensing domains. Such a structural analogy is consistent with its hypothesized role in detecting small molecules or osmotic stimuli and aligns with previous reports linking DUF4118-containing proteins to membrane-associated signal transduction [60, 79–81]. Concurrently, the GAF domain displays high-confidence structural homology to the *TePixJ* photoreceptor, supporting its role as a canonical bilin-binding photoreceptor. The conservation of photoactive residues, including the DPCF motif and the dual cysteines (C326, C354), parallels the PVB-binding chemistry seen in *TePixJ* [3, 63]. This structural conservation underscores the evolutionary retention of critical functional elements necessary for chromophore accommodation and light-dependent conformational dynamics. Collectively, these features confirm that DPCF-2 type DUF4118_CBCRs embody a structurally coherent class of photoreceptors that integrate environmental light and chemical cues into a unified histidine kinase-based signaling system, an architecture that may confer heightened adaptive plasticity in ecologically constrained cyanobacterial niches.

The dark-adapted Pb state of DPCF-2-type CBCRs is expected to adopt a doubly-linked configuration in which the bilin is covalently bound at both the canonical A-ring position (via the GAF Cys) and at C10 (via the second Cys), as established for related DXCF-lineage CBCRs [13, 25, 29]. Addition of the second thiol at C10 disrupts the π -conjugation across the bilin and yields a protonated neutral chromophore that does not show pronounced pH-dependent spectral changes within the range over which the protein remains folded [25, 29]. For this reason, we did not pursue pH titration of the Pb state, as it is unlikely to provide further mechanistic insight into the photocycle of DPCF-2 CBCRs. While our study confirms bilin binding in DPCF-2

CBCRs, additional crystallographic and spectroscopic studies are necessary to elucidate the precise chromophore interactions and protonation states. The physiological role of DPCF-2 CBCRs remains speculative. Future studies should involve in vivo expression in cyanobacterial strains to assess their role in light-driven cellular responses. The kinetic properties, quantum yield, and reversibility of the DPCF-2 photocycle should be analyzed in greater detail to determine its efficiency and potential photostability under varying light conditions. The molar extinction coefficients of the Pb and Pt states were not derived in the present study because the bound chromophore is a mixed PVB/PCB population rather than a single species; deconvolution of the contributing extinction coefficients will require either a homogeneously loaded preparation or a multi-wavelength illumination strategy. Sequential illumination with an additional yellow-to-orange light source (~ 580–600 nm) could differentially populate the PCB and PVB sub-populations, as previously demonstrated for NpF1883g3 and would enable separation of their photochemical contributions in future work [30]. Such measurements will be important for quantitative comparisons with related blue-teal CBCRs such as Tlr1999/SesB and NpF1883g2/3/4 [76]. In addition, full-length expression and systematic characterization of the individual domains of DUF4118_CBCR would further enhance its potential as an optogenetic tool. The development of DPCF-2-based optogenetic systems in heterologous hosts (e.g., *E. coli*, cyanobacteria, yeast, and mammalian cells) will provide valuable insights into their usability as light-controlled gene regulators.

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Data availability All data supporting the findings of this study are included in the article and its supplementary materials. All identifiers used throughout the manuscript, figures, and Appendix S1 are NCBI GenBank protein accession numbers; corresponding entries can be retrieved directly via the NCBI Protein database (<https://www.ncbi.nlm.nih.gov/protein/<accession>>). The complete set of protein sequences used in this study is provided in Appendix S1. The full-length DPCF-2 type DUF4118_CBCR characterized in this work is available at <https://www.ncbi.nlm.nih.gov/protein/QIR35741.1>. AlphaFold3 structural models were generated using the AlphaFold Server (<https://alphafold-server.com>) using the amino acid sequence of QIR35741.1 in FASTA format as input and can be regenerated by readers.

Declarations

Conflict of interest The authors declare no competing interests.

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