Spectral and photochemical diversity of tandem cysteine cyanobacterial phytochromes

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Abstract

The atypical trichromatic cyanobacterial phytochrome \( \textit{NpTP1} \) from \textit{Nostoc punctiforme} ATCC 29133 is a linear tetrapyrrole (bilin)-binding photoreceptor protein that possesses tandem-cysteine residues responsible for shifting its light-sensing maximum to the violet spectral region. Using bioinformatics and phylogenetic analyses, here we established that tandem-cysteine cyanobacterial phytochromes (TCCPs) compose a well-supported monophyletic phytochrome lineage distinct from prototypical red/far-red cyanobacterial phytochromes. To investigate the light-sensing diversity of this family, we compared the spectroscopic properties of \( \textit{NpTP1} \) (here renamed \( \textit{NpTCCP} \)) with those of three phylogenetically diverged TCCPs identified in the draft genomes of \textit{Tolypothrix} sp. PCC7910, \textit{Scytonema} sp. PCC10023, and \textit{Gloeocapsa} sp. PCC7513. Recombinant photosensory core modules of \( \textit{ToTCCP} \), \( \textit{ScTCCP} \), and \( \textit{GlTCCP} \) exhibited violet-to-blue-absorbing dark states consistent with dual thioether-linked phycocyanobilin (PCB) chromophores. Photoexcitation generated singly-linked photoproduct mixtures with variable ratios of yellow-orange and red-absorbing species. The photoproduct ratio was strongly influenced by pH and by mutagenesis of TCCP- and phytochrome-specific signature residues. Our experiments support the conclusion that both photoproduct species possess protonated 15\( E \) bilin chromophores, but differ in the ionization state of the non-canonical “second” cysteine sulfhydryl group. We found that the ionization state of this and other residues influences subsequent conformational change and downstream signal transmission. We also show that tandem-cysteine phytochromes present in eukaryotes possess similar amino acid substitutions within their chromophore-binding pocket, which tune their spectral properties in an analogous fashion. Taken together, our findings provide a roadmap for tailoring the wavelength specificity of plant phytochromes to optimize plant performance in diverse natural and artificial light environments.

Introduction

Phytochromes are linear tetrapyrrole (bilin)-based photoreceptors widely distributed in bacteria, algae, fungi, diatoms, and land plants that function as photoswitches to optimize photosynthesis, regulate growth and development and/or entrain behavior with the diurnal light-dark cycle (1-4). Phytochromes possess a conserved multidomain N-terminal photosensory core module (PCM) consisting of PAS (Period/Arnt/Single-minded), GAF (cGMP phosphodiesterase/Adenylyl cyclase/FhlA), and PHY (phytochrome-specific) domains associated with variable C-terminal output modules, the most prevalent functioning as two-component histidine kinases. Prototypical phytochromes transduce red (R) and far-red (FR) light signals via reversible photointerconversion between R-absorbing Pr and FR-absorbing Pfr states (2). Light absorption by phytochromes triggers photosomerization of the 15,16-double bond of their bilin chromophores (5, 6) that initiates changes in the output module structure to promote downstream signal transduction. Despite the well conserved PCM architecture of phytochromes from bacteria to land plants, various lineages have undergone evolutionary changes for sensing shorter wavelengths of light. Small spectral variation is mostly attributed to differences in linear tetrapyrrole (bilin) chromophore composition. Phytochromes leverage three bilin chromophore precursors, i.e. biliverdin (BV) and the more reduced phytobilins, phycocyanobilin (PCB) and phytochromobilin (P\( \Phi \)B), which afford modest spectral tuning within the R and FR region (7). Phytochrome chromophores are thioether linked to conserved cysteine (Cys) residues located in GAF domains of phytobilin-based plant and cyanobacterial phytochromes or in the N-terminal PAS domains of BV-based bacterial, diatom and fungal phytochromes (8-14). More drastic blue shifts have been identified in cyanobacterial and eukaryotic algal phytochrome lineages, as well as in the distantly related GAF-only cyanobacteriochrome (CBCR) lineage; such shifts arise from additional Cys residues that form thioether linkages to their bilin chromophores (15-18).

Multiple dual-Cys CBCR lineages have been characterized to date (15, 16, 19-26). However,
the tandem-Cys trichromatic phytochrome NpTP1 encoded by gene locus NpunF1183 from *Nostoc punctiforme* ATCC 29133 is the only dual-Cys cyanobacterial phytochrome to have been investigated at the biochemical level (15). NpTP1's violet-blue (VB)-absorbing Pvb dark state reflects a thioether linkage to the C10 methine bridge of its bilin prosthetic group that splits the tetrapyrrole π-conjugated system into two dipyrrole chromophores (15). NpTP1's forward photocycle is initiated by V-light, which triggers 15Z-to-15E photoisomerization of the C15 double bond and subsequent destabilization of the C10 thioether linkage. Thioether cleavage generates an orange (O) light-absorbing Po intermediate that incompletely matures into a R-absorbing Pr photoproduct over a period of minutes. The reverse photocycle involves light-triggered 15E-to-15Z isomerization followed by reformation of the C10 thioether linkage that regenerates the Pvb dark state (Fig. 1A).

The number of Tandem-Cysteine Cyanobacterial Phytochrome (TCCP) candidates has increased considerably since the initial discovery of NpTP1, which we herein rename NpTCCP. TCCPs can be recognized by a conserved 'second' Cys residue immediately adjacent to the 'canonical' Cys site of phytobilin attachment in plant and cyanobacterial phytochrome lineages, and also share the photosensory core domain architectures of both phytobilin-binding Cph1/CphA and BV-binding BphP/CphB families of cyanobacterial phytochromes. Through biochemical and spectroscopic analysis of three phylogenetically diverged TCCPs identified from *de novo* draft genomes of *Tolypothrix* sp. PCC7910 (*To*TCCP), *Scytonema* sp. PCC7513 (*Sc*TCCP) and *Gloeocapsa* sp. PCC7513 (*Gl*TCCP), we resolve TCCPs as a monophyletic clade of VB-sensing cyanobacterial phytochromes that are distinct from the well characterized R/FR cyanobacterial phytochromes. Our studies reveal unexpected spectral diversity in this dual-Cys cyanobacterial phytochrome family. From site-directed mutagenesis of ToTCCP, pH-dependent spectroscopic measurements and comparative analysis of tandem-Cys phytochrome sequences from eukaryotic algae (TCEPs), these investigations provide new insight into the structural basis of spectral tuning in the TCCP family, and the potential mechanistic roles of TCCP-signature residues in the novel photocycle of these V- to B-absorbing phytochromes.

**Results**

Multiple sequence alignments define a well-supported monophyletic TCCP lineage distinct from other cyanobacterial phytochrome families

A multiple sequence alignment of representative cyanobacterial TCCP, Cph1/CphA and cBphP/CphB phytochrome families revealed the tandem-Cys and four other highly conserved residues within the PCB-binding GAF domain as defining hallmarks of the TCCP family (Fig. S1A, blue-colored residues). Leveraging a more comprehensive alignment (Supplementary Data File 1), we resolved a monophyletic TCCP lineage, well-supported by bootstrap analysis and distinct from a polyphyletic group of Cph1/CphA and cBphP/CphB sequences (Fig. 2). Similar to SyCph1, the vast majority of TCCPs possess two component histidine kinase output domains (27) and occur in operons along with a CheY-like response regulator. The lone exception is the sensor from *Nostoc minutum* NIES26 that lacks the PHY domain as well as the canonical GAF-domain phytochrome Cys, i.e. Cys259 in SyCph1 (blue labeled TCCP in Fig. 2). TCCPs retain most of the conserved chromophore-interacting GAF-domain residues found in Cph1 and BphP families (Figs. 1B and S1A). These include Arg172, Tyr176, Tyr198, Tyr203, Asp207, Arg222, Arg254, Tyr263 and His290 (*SyCph1* numbering) - residues known to be critical to the structure and absorption properties of phytochromes. TCCP-signature residues in the GAF domain include Cys258, His262, Leu/Ile/Met270, Val272 and Ser286 (*ToTCCP* numbering, blue-colored residues in Figs. 1B and S1A). These replace residues highly conserved in Cph1 and BphP families, i.e. His260, Leu264, Ser272, Thr274 and Ala288, respectively (*SyCph1* numbering, shown in black in Fig. 1B). GAF domain residues with more variable substitutions in the TCCP family correspond to Val254, Ala256 and Gly268 (*ToTCCP* numbering, shown in green in Fig. S1A). The following studies were undertaken to address the
hypothesis that these signature residues are important to TCCP spectral tuning.

TCCPs possess violet-blue absorbing dark states with spectrally diverse photoproducts

To determine chromophore binding specificity, we expressed recombinant PCMs of ToTCCP, ScTCCP and GfTCCP in Bv-, PCB-, or PbB-producing E. coli strains. The absorption spectra of the purified proteins were consistent with formation of covalent linkages to the bilin C10 methine bridge (Fig. 3A-F). All three TCCPs possessed VB-absorbing (Pvb) dark states with absorption maxima ranging from 401 to 435 nm (Table 1, Fig. 3A-C blue-colored spectra) similar to dual thioether adducts of NpTCCP (15). Zinc-dependent fluorescence imaging of SDS PAGE gels (zinc blots) also indicated that the PCB and PbB phytobilin chromophores of ToTCCP, ScTCCP and GfTCCP remain covalently attached after denaturation (Fig. 3G). Like those of NpTCCP (15), BV adducts of ToTCCP and ScTCCP are unstable to denaturation, indicating that their A-ring thioether linkages (if present) in addition to their C10 linkages are chemically labile (Fig. 3G). By contrast, all three bilin adducts of GfTCCP were more stable to denaturation and bilin incorporation was reduced compared with ToTCCP and ScTCCP. Based upon the difference spectra, the extent of photoconversion of the PCB adducts were greater than those of PbB and BV adducts of all three TCCPs (Table 1, Fig. 3D-F). This supports the assumption that PCB is the preferred chromophore precursor of TCCPs since PbB also is not produced by any known cyanobacteria and BV binding to the canonical Cys is rarely observed.

For PCB and PbB adducts, we confirmed that light-induced photoisomerization had occurred by measurements of their photoactivity upon denaturation (Fig. S2A-C). In the native state, none of three new TCCPs showed trichromatic cycles. The 15E photostate of the PCB adduct of ToTCCP possessed both R-absorbing 15EPr (λ_{max} 662 nm) and O-absorbing 15E Po (λ_{max} 600 nm) components (Fig. 3A&D), similar to NpTCCP (15). However, formation of the R-absorbing 15E species for ToTCCP was much faster than that for NpTCCP, which exhibits trichromatic photoproduct formation. Trichromatic behavior also was not seen for ScTCCP or GfTCCP. ScTCCP yielded a single yellow orange (YO) absorbing 15E Pyo photoproduct with absorption maxima in the YO region, whereas GfTCCP yielded a mixture of both 15E Pyo and 15E Pr photoproducts (Fig. 4, and Table 1). The observed photocycles for all three TCCPs are consistent with the model that 15Z-to-15E photoisomerization of the V-B-absorbing dark state chromophores destabilizes the C10-Cys linkage initially to yield a significantly red-shifted 15E Pyo photoproduct along with variable amounts of a 15E Pr photoproduct, which sometimes appears over a long time (see conceptual model in Fig. 1A).

Cysteine mutagenesis reveals Cys258 to be responsible for violet-blue absorption of TCCP dark states, unmasking a latent G/R photocycle for ToTCCP

Owing to its robust expression, we chose ToTCCP for detailed mutagenesis studies to identify residues important for spectral tuning of its PCB adduct. Two variants of the canonical cysteine, i.e. C257A and C257S (ToTCCP numbering), retained their VB-absorbing dark states although neither was photoactive (Fig. 4A&B). This result showed that C257 was not needed for thioether linkage formation at the bilin C10 position. The C257A variant also was poorly chromophorylated, suggesting that the C257 thioether linkage at C3 improves C10-linkage formation to C258 similar to experiments performed for NpTCCP (15). Since C10 thioether linkages of bilins are chemically labile in solution, we examined whether stable linkages were retained in C257A and C257S variants following denaturation and zinc blot analysis. As expected, zinc blots for both C257 variants were negative (Fig. S3). Taken together, these results indicate that C258 is sufficient for C10 linkage formation and for retention of the VB absorption of ToTCCP's dark state.

We next examined ToTCCP variants of the TCCP-signature cysteine, i.e. Cys258 (ToTCCP numbering). These included C258H, C258S, C258D, C258I, C258F and C258Y - all of which yielded red-shifted dark states (Fig. 4C-H). As
expected, C258H displayed a R-absorbing 15Z dark state similar to Cph1, which has a histidine residue at this position (Fig. 4C). By contrast, C258S, C258D and C258I exhibited G-absorbing dark states with variable amounts of a R-absorbing species, while C258F and C258Y dark states displayed broad YO-absorbing envelopes (Fig. 4; Table 2). Of these Cys variants, C258S, C258D and C258I were photoactive and all exhibited atypical G/R photo cycles. By comparison, C258H, C258F and C258Y dark states were photoinactive. All C258 variants also retained stable covalent linkages as shown by zinc blotting, although the reduced zinc blot signals for C258I, C258D, C257Y and C258F are likely due to poor PCB binding (Fig. S3). These observations indicate that C258 plays an essential role in spectral tuning of the VB-absorbing dark state of ToTCCP.

In view of the robust G/R photocycle observed for the C258S variant of ToTCCP (Fig. 4D), we constructed the corresponding Cys-to-Ser variants for Gt/TCCP (C249S) and Sc/TCCP (C257S). Unlike the C258S variant of ToTCCP, Gt/TCCP (C249S) and Sc/TCCP (C257S) variants exhibited mostly R-absorbing (λmax 645 nm) or mostly green (G)-absorbing (λmax 530 nm) dark state mixtures, respectively, that strongly bleached upon light treatment (Fig. S4ABE&F and Table 2). These results show that the spectral consequences of substitution of the TCCP-signature cysteine with serine are species dependent and difficult to predict.

Highly conserved in all cyanobacterial phytochrome families (Fig. S1A), C287 and C303 in ToTCCP are located in good positions to form a disulfide linkage (28). For this reason, we also constructed C287S and C303S variants to test the importance of these cysteine residues in the ToTCCP photocycle. The spectra and photocycles of these variants were almost indistinguishable for those of the wild-type parents (compare Fig. S4CDG&H with Fig. 3A&D). Since natural variation is seen at both cysteines in all cyanobacterial phytochrome lineages, it appears that disulfide bond formation between these conserved Cys pair is not necessary for native TCCP photocycles.

**Mutagenesis of conserved TCCP species-specific residues reveals potential roles in photoproduct spectral tuning**

To identify residues affecting photoproduct tuning in the TCCP family, we exchanged representative TCCP-signature residues in ToTCCP with those found in Cph1 and BphP families. Residues initially targeted for mutagenesis included two with hydrophilic sidechains, i.e. H262 and S286 (ToTCCP numbering), and two with hydrophobic sidechains, i.e. L270 and V272 (ToTCCP numbering), which correspond to SyCph1 residues L264 located in α8, A288 located in β11, and S272 and T274 located in β10, respectively (see Figs. 1B & S1). We generated five ToTCCP variants by replacement of these four residues with those found in SyCph1 and additional variants of H262 and V272 that occurs infrequently in the TCCP family. The absorption spectra of the dark states of all variants were similar to those of the wild type (Fig. 5A-E, blue spectra; Table 2). By contrast, the photoproduct spectra of these variants all differed from that of wild-type ToTCCP (Fig. 5A-E; also see Fig. S5A-E for difference spectra). Instead of the dual-peaked spectrum of the wild type ToTCCP, photoproducts of H262L, H262Y and L270S variants exhibited R-absorbing peaks lacking the YO-absorbing shoulder. Interestingly, the photoproduct spectra of V272T and V272S variants were not identical to each other, with the former exhibiting delayed formation of a photoproduct mixture similar to NpTCCP and a single Y-absorbing peak being observed for the latter. The S286A variant also exhibited delayed formation of a photoproduct mixture similar to NpTCCP. These observations indicate that the four TCCP-conserved residues are not important for the dark state spectra, but all participate in spectral tuning of the ToTCCP photoproduct. Moreover, none of the variants examined yielded a FR-absorbing 15E-photoproduct seen in the Cph1 family. It is therefore likely that multiple changes in these conserved residues are required to further red shift TCCP photoproducts into the far red.

ToTCCP residues not well conserved in the TCCP family were also investigated, including
V254, A256, F261, and G268 (Fig. S1A, highlighted in green). We hypothesized that the trichromatic photocycle might be restored by substituting these residues with those found in the trichromatic \textit{Np}TCCP. Among the variants tested (Fig. 5G-K), only the F261W variant exhibited delayed formation of a mostly R-absorbing photoproduct similar to \textit{Np}TCCP, although V254F also yielded a R-absorbing photoproduct. By contrast, the photoproduct spectra of A256W, F261Y and G268K were quite similar to wildtype ToTCCP. These results highlight supporting roles for V254 and F261 in TCCP photoproduct spectral tuning.

We also examined \textit{To}TCCP variants in strongly conserved residues in the extended phytochrome superfamily. These included D205N, Y174H/F, H288A and Y201F, to target chromophore contacting residues known to play important roles in spectral tuning in Cph1 and BphP families. D205 corresponds to \textit{Sy}Cph1 residue D207, an invariant residue that has been proposed to function as a counterion for the cationic protonated bilin chromophore (28). D205N yielded an O-absorbing photoproduct with very little R-absorbing photoproduct (Fig. 5L). The photoproducts of both variants of Y174, i.e. Y174F and Y174H, as well as the variant H288A were also enriched in the YO-absorbing species (Fig. 5M-O). While the inclusion of imidazole in the assay buffer might influence the spectra of His variants, at 250 \textmu M it is unlikely to have a significant differential effect on TCCP variants reported here. Only the photoproduct of the Y201F variant was quite similar with wild-type \textit{To}TCCP (Fig. 5P), underlying the universal importance of the nearly invariant D205, Y174 and H290 residues in photoproduct tuning in all phytochrome lineages.

\textbf{Spectroscopic titration of \textit{To}TCCP and its singly-linked C258S variant reveals pH-dependent equilibria between R-absorbing protonated and G-absorbing deprotonated states}

To test whether the varying ratio of YO- and R-absorbing photoproducts is due to differences in ionization states of the chromophore and/or nearby amino acid residues, we undertook experiments to test the effect of pH on the \textit{To}TCCP photoproduct spectrum - an approach was used previously to examine the pH-dependence of both dark and photoproduce states of the G/R CBCR RcaE (29) and the B/O CBCR Oscil6304_2705 (26). As expected for a neutral doubly-linked chromophore (Fig. 1A), we first confirmed that the spectrum of 15Z dark state of \textit{To}TCCP was unaffected by changes in buffer pH (Fig. 6A). By contrast, the 15E photoproduce state spectrum of \textit{To}TCCP was strongly pH dependent, supporting the assignment of the YO-absorbing (579 nm) and R-absorbing (663 nm) species to deprotonated and protonated photoproducts, respectively (Fig. 6B). Based on these studies, we estimated an apparent pKa of 9.5 for the titratable group(s) responsible (Fig. S6A). pH shift measurements were also performed as a control to test whether these spectral changes were reversible after cycling of the pH from pH 10 to pH 7 and vice versa. These experiments indicated that the spectra of the fully deprotonated and fully protonated photoproduce species were identical before and after each pH shift (Fig. 6C), thereby establishing that \textit{To}TCCP is not irreversibly denatured in this pH range.

pH titrations of both photostates of the C258S variant of \textit{To}TCCP were next examined. The photocycle of this variant (Fig. 4D) is very similar to that of the G/R CBCR RcaE whose G-absorbing 15Z dark state chromophore is deprotonated and its R-absorbing 15E photoproduce chromophore is protonated (29). Like RcaE, both 15Z and 15E states of the C258S variant were pH sensitive. The G-absorbing 15Z dark state could be converted to a R-absorbing species with a peak at 635 nm and the shorter wavelength-absorbing shoulder of the 15E photoproduce could be eliminated by lowering the pH to 6 (Fig. 6D). Based on this titration experiment, an apparent pKa of 6.6 was estimated for the titratable group(s) responsible for the spectral shift of the 15Z dark state (Fig. S6B). The presence of clear isosbestic points also corroborates a two species model. We next performed a pH titration experiment with the 15E photoproduce of the C258S variant (Fig. 6E). Similar to WT \textit{To}TCCP, the pH-dependent equilibrium between R- and G-absorbing species revealed a titratable group(s) with an apparent pKa of 9.7 (Fig. S6C). pH-shift spectroscopic
measurements also indicated that titrations of the C258S variant were reversible in both directions (Fig. 6F) showing that C258S was not denatured in the pH 7-10 range.

**pH dependence of other ToTCCP variants reveals complex interplay of signature GAF-domain residues in photoproduct tuning**

To explore the influence of TCCP-signature residues on spectral tuning of the 15E-photoproduct of ToTCCP, we examined the pH-dependence of the eight previously described ToTCCP variants, D205N, V254F, F261G/W, H262L, L270S, V272T and S286A. In this experiment, each variant was examined spectrophotometrically at pH 6, 8 and 11. All variants bound PCB and exhibited YO-absorbing spectra at pH 11 (Fig. 7). With the exception of D205N, we observed an increase in the R region as the pH was lowered to pH 6 for the photoproduct 15E states of all variants (Fig. 7A). Of these variants, only V272T and S286A retained significant YO absorption at pH 6 (Fig. 7G &H). Surprisingly, the YO peak intensity of V272T increased as the pH was lowered from 11 to 6. A similar spectral change was seen for S286A except that the maximum amount of red absorbing species was fully formed at pH 8 and remained unchanged as the pH was further lowered to 6. These results suggest that a second YO species with a larger absorption coefficient was produced as the pH was lowered to 8 for the V272S and S286A variants. A similar phenomenon was seen for the V254F and F261W variants, although both second YO and R peaks increased and then partially disappeared as the pH was lowered to 8 and then to 6 (Fig. 7B & D). For these experiments, photoproduct heterogeneity was evident by the lack of clear isosbestic points in pH titrations of V254F, F261W, and V272S variants (Fig. 7B, D & G). By contrast with the appearance of the second YO species, F261G, H262L and L270S variants exhibited a stepwise direct conversion to a R-absorbing species as the pH was lowered from 11 to 6 (Fig. 7C, E & F). Clear isosbestic points were seen in F261G and H262L spectrophotometric titrations indicating the presence of two species in pH-dependent equilibrium. As controls, seven of the eight variants except the Y-absorbing D205N variant retained photoreversibility upon forward or reverse cycling from pH 7 to pH 10 buffer (Fig. S7). D205N yielded a mixture of G- and V-absorbing species at pH 10 upon photocycling to/from each photostate. This suggests that the D205N mutant is irreversibly denatured by high pH.

**PHY domain variants reveal participation of the PHY domain in the conversion between the YO- and R-absorbing photoproducts**

In single-Cys phytochromes such as Cph1, BphP and ArPHYB, PHY domain motifs WGG, PRxSF and HbXE have been shown to stabilize the 15E Pfr photoproduct state (30, 31). For example, variants in WGG (G564E) and PRxSF (R582A) motifs of phyB inhibited thermal 'dark' reversion of Pfr (31). Since these PHY domain motifs are also conserved in the TCCP family (Fig. S1B), we constructed the A451G, R471A, R471K, K472F, and E479A variants of ToTCCP to target their role in stabilizing one or both of the photoproduct states (Fig. S8). R471A and R471K variants yielded only the 15E Pyo photoproduct. All other PHY domain variants afforded final product mixtures with spectra very similar to that of the wildtype, although product maturation was considerably delayed in A451G and E479A variants. These results suggest that the photoproduct red shift is influenced by interactions with the PHY domain, consistent with studies on the formation of the Pfr state in prototypical plant and cyanobacterial phytochromes that lack the PHY domain (32).

**Discussion**

Our studies resolve a phylogenetically distinct clade of PCB-binding TCCP lineage of two-Cys cyanobacterial phytochromes, which are mostly restricted to members of the more recently evolved subsection IV and V clades of filamentous, nitrogen-fixing cyanobacteria (Fig. 2). Evolution of this group coincided with increasing atmospheric oxygen accumulation during the Great Oxidation Event (33). Filamentous growth observed in multicellular cyanobacteria would be advantageous in improving cell motility as well as metabolic fitness compared with unicellular cyanobacteria (33, 34). TCCPs sense V-to-B light and yield...
photoproducts that absorb in the YO and/or R spectral regions. Both long-wavelength-absorbing photoproducts appear after photoisomerization of the dual-linked, dark-state chromophore - a process that destabilizes the C10-thioether linkage (Fig. 1A). Our studies show that the equilibrium between YO- and R-absorbing photoproducts is pH dependent. This suggests that TCCP sensors may be able to integrate light quality and cellular pH, both of which vary throughout the diurnal light-dark cycle and from light fluctuations in the natural environment due to changes in depth or shading by neighbors. The ability to sense and utilize B-, G- and YO-light for photosynthesis is invaluable to cyanobacterial species which live at depth where R and FR light are strongly attenuated in water. This selective pressure likely was one of the factors contributing to the evolution of TCCPs as well as the more spectrally diverse CBCR family.

Aside from the tandem cysteines, TCCP-signature residues play a minimal role in spectral tuning of the 15Z dark state

Our cysteine mutagenesis studies establish that formation of the C10 thioether linkage does not require the canonical cysteine to generate the VB-absorbing 15Z dark state (Fig. 4A&B). However, the canonical cysteine is needed for photoactivity since the C257A and C257S variants are photochemically inactive. This implies a critical role for the canonical Cys linkage, e.g. for proper positioning of the bilin chromophore in the protein pocket. All variants of the TCCP-signature cysteine which bound bilin exhibited significantly red-shifted dark states with absorption maxima that varied from green to orange. This corroborates the observations of previous studies that show the TCCP-signature cysteine to be essential for dark-state tuning (15). Several of these variants, i.e. C258S, C258D and C258I, yielded G-absorbing dark states similar to those of G/R CBCRs such as FdReaE (Fig. 4D-F). Indeed, spectrophotometric titrations on the C258S variant indicate that the G- and YO-species are interconvertible via a pH-dependent process with an apparent pKa of 6.6 (Figs. 6D and S6B) - a result similar to the dark state of the G/R CBCR FdReaE (29). However, the protonated dark state species of FdReaE maximally absorbs in the red region, not in the YO region. These results support the interpretation that the G-absorbing chromophore of the C258S dark state is deprotonated, whereas the protonated YO-absorbing 15Z chromophore adopts a more distorted out-of-plane geometry that blue-shifts its absorption maximum. The C258H variant, which restores the conserved 'axial' histidine residue found in all R/FR phytochromes, also absorbs mostly in the yellow-orange region. This suggests that the C258H dark-state chromophore is fully protonated at pH 7.5, similar to phytochromes albeit with a more distorted chromophore that yields a blue-shifted absorption maximum.

Mutagenesis of TCCP-signature residues revealed little influence on 15Z dark-state tuning of ToTCCP. With the exception of variants the tandem cysteine pair, none of signature residue variants, i.e. H262, L270, V272 and S286 (ToTCCP numbering; Fig. 1B), abolished second thioether linkage formation nor appreciably altered the VB absorption of their 15Z dark states (Fig. 5A). Since both 15Z,syn and 15Z,anti configurations at C15 are compatible with a VB-absorbing dark state, it is possible that one or more of these variants could have affected the C15 configuration while not affecting dark-state absorption. Moreover, the pH has little or no effect on the 15Z dark state absorption of TCCPs - with the exception of G/TCCP whose second thioether linkage is sensitive to extreme pHs (Figs. 6 and S8). This is reasonable because the double linkage splits the PCB chromophore into two uncharged dipyrroles neither of which can donate or accept a proton in the pH 6-11 range used in this study (35).

With the exception of S286, TCCP-signature residues are predicted to be located on the α-facial side of the chromophore (Fig. 1B, residues in blue). This includes the TCCP-signature cysteine that constrains the thioether linkage to C258 to be α-facial, which contrasts with the β-facial C10 linkage found in the dual-cysteine CBCR TePixJ and related CBCRs (16, 36). The dual α-facial linkages constrain the A-B ring system to be closely appressed to the α8 helix and likely prevent binding of the α-facial pyrrole.
water found in canonical phytochromes. The α-facial C10 linkage would also require the C-D ring system to tilt towards the β-face of the chromophore pocket. This orientation would preclude H-bonding between the D-ring carbonyl and the imidazole sidechain of H288 - a highly conserved residue known to stabilize the 15Z,anti configuration of Pr chromophores in prototypical phytochromes (28, 37). The lack of this interaction in the doubly linked species may affect the equilibrium between the C15,syn and C15,anti conformations for the 15Z dark state.

The exchange of the polar residues in the β10 strand in Cph1, i.e. S272 and T274, with nonpolar residues in TCCPs, i.e. L270 and V272 in T0TCCP, also ensures that polar interactions with the bilin propionates do not occur. The relatively non-polar pocket of TCCPs compared with Cph1/BphP families as well as the neutral charge of the doubly linked chromophore may also influence the ionization state of one or both propionic acid sidechains of the 15Z dark state chromophore. For this reason, we hypothesize that the B-ring propionic acid is protonated and neutral in the 15Z dark state as depicted in Fig. 1A. Based on modeling of the T0TCCP dark state using DrBphP and SyCph1 dark-state structures as templates (PDB IDs 2O9B and 2VEA), we propose that the β-facial serine S286 is located in a good position to stabilize the 15Z,anti configuration of the dark state chromophore via H-bonding with the D-ring carbonyl (see Figs. 1B and S1A). However, this hypothesis remains to be tested. Since the B-absorbing dark state absorption is not expected to change much by the C15 syn-to-anti conversion, the 15Z,syn configuration of the T0TCCP dark state at present remains a formal possibility.

**pH plays a critical role in the 15EPr:15EPr photoproduct equilibrium**

By contrast with dual-linked dark states, the TCCP photoproduct equilibrium is strongly affected by pH. For nearly all wild-type and variant TCCPs studied, we observed a mixture of YO- and R-absorbing photoproducts whose ratio is affected by both pH and signature residue substitutions. Primarily based on evidence that the D205N variant of T0TCCP adopts an O-only photoproduct state at all pHs (Fig. 7A), we initially favored assignment of the O- and R-absorbing photoproducts to species with deprotonated-neutral and protonated-cationic π systems, respectively. The G/R photocycle of the C258S mutant also is consistent with the hypothesis that the initial 15E-photoproduct of wild-type T0TCCP would possess a neutral deprotonated bilin chromophore and a neutral protonated C258 sulfhydryl sidechain. However, such a neutral state would equilibrate with a zwitterionic 15EPr state with a protonated cationic 15E,anti bilin system and an anionic sulfhydryl on C258 as shown in Fig. 1A. We also hypothesize that the H262 sidechain is located in a good position to stabilize this sulfhydryl anion upon transfer of a proton from the B-ring propionic acid to generate a imidazolium cation that bridges the propionate and sulfhydryl anions as depicted in lit State 1 in Fig. 1A.

Since acid-denatured biliproteins with 15E bilin chromophores absorb YO rather than G light (29, 38, 39), we favor the interpretation that the 15EPr photoproduct possesses a protonated bilin chromophore at pH 8, also consistent with the zwitterionic Lit State 1 structure. Although low pH favors conversion of Lit State 1 to Lit State 2 by protonating the C258 sulfhydryl, which would disrupt its ion-pair interaction with the protonated chromophore, this interconversion likely involves more substantive changes in the interactions between the GAF and PHY domains. We therefore hypothesize that the observed ratio of 15EPr to 15EPr photoproducts represents the equilibrium between two protonated chromophore states whose energetics reflect distinct networks of pH-dependent H-bonding, ion-pair and steric interactions. Since our spectral titration studies reveal an 'apparent' pKa of 9.5 for the conversion between the two 15E photoproducts of wild-type T0TCCP (Fig. S6C), we tentatively assign this equilibrium to the ionization of the C258 sulfhydryl group.

We envisage the 15EPr and 15EPr conversion to be accompanied by significant changes in chromophore-protein interactions upon sulfhydryl protonation, such as counterion exchange for both propionate ion pairs, disruption of the D205-R471 ion pair between GAF and PHY domains, restructuring of the secondary structure of the tongue motif as well as
formation of new H-bonding and ion pair interactions between protein residues and the bilin chromophore (30, 42, 43). Hence, the pKa estimate from titration experiments likely entails contributions from multiple weak acid species that affect the kinetics and thermodynamics of this interconversion. Moreover, TCCP photoactivation also is expected to influence intersubunit interactions in the full-length homodimer and those of the truncated TCCP photosensory modules studied here that appear to be dimers by native gel electrophoresis and steric exclusion chromatography. Thus, the level of chromophorylation might play a critical role in stabilizing the fully activated state of the TCCPs. Since these intersubunit interactions are likely to be pH dependent, the variable $^{15}$E$^\text{Pyo}$ to $^{15}$E$^\text{Pr}$ ratios for the four TCCPs examined here and previously (15) also may be influenced by different ratios of partially and fully chromophorlated dimers. Indeed, recombinant TCCPs from $E. \text{coli}$ are poorly chromophorlated. Hence, it is conceivable that the variable 'equilibria' between the two photoproduct states of TCCPs arises from different ratios of partially and fully chromophorlated species that affects the relative energetics of the two photoproduct proteins.

It is well established that both $15E$ and $15Z$ chromophores of Cph1 are inherently heterogeneous (44-47). Such heterogeneity might be due to $15,\text{syn}$ vs. $15,\text{anti}$ chromophore configurations, $\alpha$- vs. $\beta$-facial dispositions of the D-ring, A-ring out-of-plane twisting, alternative protein-chromophore H-bonding networks, and/or relaxed vs. trapped twist conformations of the D-ring - any of which are formal possibilities for the pH-dependency of the $^{15}$E$^\text{Pyo}$:$^{15}$E$^\text{Pr}$ photoproduct ratio. Since the chromophore pocket of dark states of phytochromes - and those of TCCPs by analogy - are relatively solvent inaccessible, proton transfer rates from bulk solvent to the chromophore could be quite impaired, requiring re-orientation of proton donor and proton acceptor groups, including water, that may be strongly constrained within the chromophore binding pocket and requiring disruption and reformation of strong secondary interactions like those observed following bacteriophytochrome $D_rBphp$ photoactivation that have a formidable energetic barrier to accomplish (48).

**TCCP-signature residues strongly influence the $^{15}$E$^\text{Pyo}$:$^{15}$E$^\text{Pr}$ photoproduct equilibrium**

Our mutagenesis studies corroborates the interpretation that protonation state of the TCCP-signature cysteine plays a dominant role in the $^{15}$E$^\text{Pyo}$:$^{15}$E$^\text{Pr}$ equilibration process. Compared with wildtype at pH 8, the C258S variant exhibits an enhanced yield of the R-absorbing $^{15}$E$^\text{Pr}$ photoproduct (Fig. 4D). Isosteric with Cys, Ser cannot be deprotonated to stabilize a YO-absorbing ion pair with the protonated bilin system, thereby favoring the $^{15}$E$^\text{Pr}$ photoproduct. By contrast, the C258D variant favors the $^{15}$E$^\text{Pyo}$ photoproduct (Fig. 4E), that could be rationalized by its low pKa and difficulty to protonate in situ. However, it is also possible that the aspartate variant could interact with nearby cationic residues, e.g. H262, R220 or R254, to inhibit the interconversion to the Lit State 2. Substitution of C258 with amino acids with larger sidechains, i.e. C258L, C258L and C258Y, affected both the yield of chromophore attachment and the photochemical activity (Fig. 4F-H). This suggests that these variants alter the positioning of the bilin chromophore possibly to minimize unfavorable steric interactions. In view of the altered photoproduct equilibrium of the Y174F and Y174H variants, it is also possible that Y174 anion formation can destabilize the R-absorbing species at high pH possibly by disruption of an H-bond between the ionized C-ring propionate observed in the $^{15}$E$^\text{Pfr}$ form of bacteriophytochromes (42). However, this interpretation cannot be responsible for other mutations that favor the $^{15}$E$^\text{Pyo}$ species, i.e. D200N and H288A. Until the crystal structures of the $^{15}$E$^\text{Pyo}$ and $^{15}$E$^\text{Pr}$ photoproducts are resolved, we favor the interpretation that the C258 thiol ionization is the primary determinant for regulating the ratio between the two photoproduct states (Fig. 1A).

In addition to the C258S variant, variants H262L, H262Y, L270S, V254F and F261W all exhibited increased amounts of the $^{15}$E$^\text{Pr}$ photoproduct (Fig. 5A-C, G and J). We envisage that these variants destabilize the $^{15}$E$^\text{Pyo}$ photoproduct possibly by increased steric clashes or by gain/loss of H-
bonding interactions with the bilin propionate sidechains. Indeed, most of these variants restore residues found in phytochromes that stabilize the $^{15E}$Pfr photoproduct state. We also observed that some of the ToTCCP variants slow down the conversion between the two states without altering the final equilibrium, i.e., V272T, S286A and F261W (Fig. 5D, E and J), similar to the photocycle of NpTCCP (15). We attribute the effect of these variants to an increase in the energetic barrier between the $^{15E}$Pyo to $^{15E}$Pr conversion that likely alters the rearrangement dynamics of the PHY tongue region following photoisomerization seen in cyanobacterial, plant and (bacterio)phytochromes (30, 31, 48).

Moreover, R471A or R471K variants favor the $^{15E}$Pyo photoproduct, indicating that this D205-contacting residue found in the PHY domain also plays an important role in photoproduct tuning in ToTCCP. Whereas none of the other variants in PHY domain, i.e., A451G, E479A and K472F, appreciably influenced the photoproduct ratio, a minor role of these residues in ToTCCP photoproduct tuning.

**Tandem-Cys phytochromes also have also evolved in the eukaryotic algae**

While short-wavelength-absorbing streptophyte alga and land plant phytochromes so far have not been identified, B-, G-, Y- and O-sensing phytochromes have diversified in eukaryotic algae (17). Evidence for independent evolution of tandem-cysteine phytochromes has been reported in glaucophyte alga, the most ancient eukaryotic algal lineage (17). The present studies suggest that a small number of amino acid substitutions within the GAF domain may be sufficient to transform a Cph1 (or BphP) into a fully photoswitchable TCCP variant. These substitutions correspond to four signature positions, H262, L270, V274, and S286 (ToTCCP numbering; Fig. 1B), plus the ‘tandem’ Cys sites, C257 and C258. Examination of eight tandem-Cys phytochrome sequences from glaucophytes currently present in publicly available databases also supports this hypothesis; all six signature positions for these TCEPs either possess the same amino acids found in ToTCCP, conservative amino acid substitutions and/or variants present in one or more cyanobacterial TCCP representative (see Supplementary Data File 1). Attempts to multiplex all of these TCCP-signature residues into the SyCph1 scaffold to secure a variant with a robust TCCP photocycle so far have been stymied by low yields of recombinant protein. Additional mutagenesis to sustain protein folding of SyCph1 and other phytochrome candidates may be needed.

**A roadmap towards spectral diversification of plant phytochromes**

Amongst the more exciting discoveries of this work is the generation of the C258S variant of ToTCCP that has a G/R photocycle similar to RcaE. Although the same residue exchange in other TCCPs failed to generate variants with robust G/R photocycles, this suggests that it will be possible to generate variants of plant phytochromes with a broad range of photocycles, considerably extending their R/FR photosensory range. By introducing these variants into plant phyA or phyB photoreceptors, we hope to examine how changes in wavelength sensitivity will influence plant performance (49, 50). Such phytochrome variants could prove better than their wild-type counterparts for sustaining robust growth and development in artificial light environments that lack sufficient sunlight.

Towards the future, one pressing question left to address is the structural basis for FR-absorption of cyanobacterial and algal phytochromes that use PCB as chromophore precursor. Despite our efforts to reintroduce residues found in the chromophore binding pockets of Cph1, Cph2 and algal phytochromes, we have yet to secure a variant of ToTCCP whose photoproduct absorbs in the far red. We believe that the cysteine variant C258H, which possesses a photoinactive R-absorbing dark state, is a good place to start these investigations. By introducing other variant amino acid substitutions, both targeted and random, such studies are expected to provide valuable insight into the molecular basis of FR-absorption by plant, algal and cyanobacterial phytochromes.

**Experimental procedures**

Expression and purification of recombinant His-tagged photosensory core modules (PCMs)
of TCCPs from BV-, PCB- or PΦB producing Escherichia coli cultures

Plasmids pPL-BV, pPL-PCB, and pPL-PΦB were used for BV, PCB and PΦB biosynthesis in E. coli (51). PCMs of TCCPs, TpToTCCP (Tpr0787, GenBank accession MG345012), ScTCCP (Spr6403, GenBank accession MG345011) and GtTCCP (Gpl2095, GenBank accession MG345010), were amplified by PCR with appropriate primers (Supplementary Table S2) with genomic DNA from Tolypothrix PCC 7910, Gloeocapsa PCC 7513, and Scytonema PCC 10023 (axenic strains and genomic DNAs provided by M. Gugger, Collection of Cyanobacteria of the Institute Pasteur) as templates (52-54). PCR fragments were digested with appropriate restriction enzymes and then cloned into the pBAD-MycHisC vector (Invitrogen, USA) to yield pBAD-TCCPPCM bacterial expression plasmids for various TCCPs. All constructs were verified by oligonucleotide sequencing. E. coli LMG194 (Invitrogen) cells co-transformed with pBAD-TCCPPCM and pPL-BV, -PCB, and -PΦB were grown overnight at 37°C in 5 ml of minimal medium RM (55) containing 50 μg/ml kanamycin and 200 μg/ml ampicillin. Recombinant PCM proteins were isolated and further purified by nickel affinity chromatography (Qiagen) as previously described (52). Eluted proteins in elution buffer [50 mM Tris-HCl pH 8, 300 mM NaCl, and 250 mM imidazole] were desalted and concentrated to yield a final concentration of 300 µM NaCl and 250 µM imidazole in 50 mM Tris-HCl buffer (pH 8) using an Amicon Ultra Centrifugal filter (Millipore). For pH titration experiments, 30 µl of each protein sample in distilled water adjusted pH 8 was transferred to the tube containing 120 µl of the designated 1 M stock buffer. Spectra were recorded immediately after sample mixing by gentle pipetting (29). To estimate pK<sub>a</sub> values for 15E ToTCCP samples, the absorbance at 662 nm was plotted against the pH and then fitted to a hyperbolic equation (OriginPro 8.1; 57). pKa values for other TCCP samples were determined similarly using appropriate irradiation wavelengths and peak absorbances.

Spectrophotometric analyses

Steady-state absorbances of purified TCCPs were recorded at room temperature with a UV1601 spectrophotometer (Shimadzu, Japan) in standard assay buffer (50 mM Tris-HCl, pH 8) or after dilution with buffers of varying pH from 5 to 11 as described below. Forward and reverse photointerconversions were triggered in the transparent tube using a UV-A tube (Model XX-40, Spectroline, 355 ± 28.6 nm) or blue or red LED lamps (Sungkwang LED Co., 525 ± 15 or 660 ± 15). Incident photon fluxes for UV-A (70 µW cm<sup>-2</sup>), blue (52.8 µmol m<sup>-2</sup> s<sup>-1</sup>) and green (15.9 µmol m<sup>-2</sup> s<sup>-1</sup>) lights for forward and reverse photoconversions were illuminated for 1 min and dark 15E to 15Z reversion was undetectable for any sample.

pH titration assays

Fifteen µl of different buffers were added into transparent tubes containing 135 µl of the purified TCCP samples (ca. 2.8 ~ 4.7 µg) in either 15Z dark state or 15E lit state after saturating irradiation with blue light. Buffers included 1 M MES-NaOH for pH 5 and 6, 1 M Tris-HCl for pH 7, 8, and 9, 1 M glycine-NaOH for pH 10 and 11, and 1 M KCl-NaOH for pH 12 and 13. After irradiation, all sample manipulation was performed in darkness. For pH-jump experiments, 30 µl of each protein sample in distilled water adjusted pH 8 was transferred to the tube containing 120 µl of the designated 1 M stock buffer. Spectra were recorded immediately after sample mixing by gentle pipetting (29). To estimate pK<sub>a</sub> values for 15E ToTCCP samples, the absorbance at 662 nm was plotted against the pH and then fitted to a hyperbolic equation (OriginPro 8.1; 57). pKa values for other TCCP samples were determined similarly using appropriate irradiation wavelengths and peak absorbances.

SDS-PAGE and in-gel zinc-dependent fluorescence assays

For zinc in-gel fluorescence analysis (58), purified TCCPs (3 µg) were separated on a 12% (w/v) SDS-PAGE gel. Gels were then soaked in unbuffered 20 mM zinc acetate at room temperature for 30 min in the dark, and then imaged for fluorescence under UV-B excitation (302 nm) using a Bio-Rad Gel Doc 2000 equipped with a blue bandpass filter (480BP). Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and reimaged to determine
abundance of each polypeptide using a Bio-Rad ChemiDoc imager. Concentrations of thioether-bound 15Z-PCB and 15Z-PΦB were calculated after denaturation in 8M urea adj ust to pH 2.0 with HCl using extinction coefficients 35.5 mM$^{-1}$ cm$^{-1}$ at 663 nm (59, 60) and 32.1 mM$^{-1}$ cm$^{-1}$ at 660 nm (60, 61), respectively. Protein concentrations were then estimated on the denatured samples using the calculated molar extinction coefficient ($\varepsilon$) at 280 nm provided by the ExPASy ProtParam tool (https://web.expasy.org/protparam/) based on the 6xHis-tagged polypeptide sequences (ToTCCP, 63,870 M$^{-1}$ cm$^{-1}$; ScTCCP, 56,880 M$^{-1}$ cm$^{-1}$ and G/TCCP, 59,735 M$^{-1}$ cm$^{-1}$) and correcting for protein absorption at 280 nm. When expression levels were low, relative protein concentrations were determined using BioRad’s Quantity OneTM software. Chromophorylation yields of acid denatured TCCP samples were determined by dividing the concentration of the bilin by the concentration of the protein (62).

## Bioinformatics and phylogenetic reconstructions

Phytochrome protein sequences SyCph1, CaCphB/BphP and ToTCCP (Tpr0787) were used for BlastP analysis as query sequences and yielded 416 protein sequences from 174 cyanobacteria genomes and 85 protein sequences from 68 bacterial genomes (E $\leq$ 1 $\times$ 10$^{-10}$). TCCP, Cph1/CphA, and BphP/CphB sequences were summarized by cyanobacteria genera and subsections, and the number of proteins by strains was determined (and plotted in Supplementary Table S1). For phylogenetic reconstructions, multiple amino acid sequence alignments were carried out using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). For phylogenetic tree construction of cyanobacterial phytochromes we collected the submitted phytochrome related sequences of bacteria, yeast, glaucophytes, and streptophytes from NCBI. We used the predicted PAS, GAF and PHY domain sequences for reducing the discriminant disorder resulting from the interspecies variations of N-terminal sequences. The total of 46 amino acid sequences was involved in the dataset and aligned using ClustalW in MEGA7 (64). The maximum likelihood (ML) tree was constructed based on the best substitution model which was selected the LG+$\Gamma$I model (63) with Gamma distribution (5 categories; +$\Gamma$, parameter=1.318) and Invariable site (+$I$, 2.02% sites) in MEGA7 (64). The bootstrap value was estimated using 1,000 replicates with the same substitution model. Thick branches in phylogenetic tree indicated >80% of the ML support value. Homology modeling of the PCM of ToTCCP was performed online (https://swissmodel.expasy.org/) using default parameter settings and the 15E state of agp2-PAiRFP2 (6G1Z) as template.

## Data availability:
All the data are contained within the manuscript and the associated supporting information files. Proteins presented in this paper have been deposited in GenBank with following IDs: MG345010, MG345011, MG345012, and MG811563.

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## Conflict of Interest:

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The authors declare that they have no conflicts of interest with the contents of this article.

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**Footnotes**

The abbreviations used are: BphP, bacterial phytochrome; BV , biliverdin; CBCRs, cyanobacteriochromes; Cph, cyanobacterial phytochrome; GAF, cGMP phosphodiesterase/Adenylyl cyclase/FhlA domain; PAS, Period/Arnt/Single-minded domain; PCB, phycocyanobilin; PCM, photosensory core module; Φ B, phytochromobilin; PHY, phytochrome-specific domain; TCCPs, tandem cysteine cyanobacterial phytochromes; TCEP, tandem cysteine eukaryotic phytochrome; color codes are near-UV (U), 300−394 nm; violet (V), 395−410 nm; blue (B), 411−485 nm; teal (T), 486−514 nm; green (G), 515−569 nm; yellow (Y), 570−585 nm; orange (O), 586−614 nm; red (R), 615−685 nm; far-red (FR), 685−760 nm and near-IR, 761-1000 nm.
Table 1. Absorption spectral parameters for TCCP sensors with different bilin chromophores.α

| Protein | Bilin | λmax | SAR | Yc | %ΔΔA |
|---------|-------|------|-----|----|------|
| TyTCCP  | BV    | 411;424 | 0.22* | na | 47.5(0.287) |
|         | PCB   | 414;662 | 0.32 | 0.611 | 100(0.604) |
|         | ΦB    | 435;615 | 0.25 | 0.502 | 91.4(0.552) |
| ScTCCP  | BV    | 401;401 | 0.26* | na | 27.1(0.181) |
|         | PCB   | 401;571 | 0.23 | 0.385 | 100(0.667) |
|         | ΦB    | 414;594 | 0.10 | 0.176 | 45.9(0.306) |
| G/TCCP  | BV    | 426;607 | 0.07* | na | 28.2(0.072) |
|         | PCB   | 408;581 | 0.10 | 0.117 | 100(0.256) |
|         | ΦB    | 429;598 | 0.02 | 0.024 | 44.5(0.114) |
| NpTCCP  | PCB   | 392;598,670 | 0.22 | na | 85.0 |

α Recombinant photosensory core domain (PAS-GAF-PHY) constructs were purified after expression in E. coli cultures synthesizing BV, PCB or ΦB (from Figs. 3 and S2) and resuspended 50 mM Tris-HCl buffer (pH 8) containing 12 mM NaCl and 10 mM imidazole.

β Peak wavelengths are reported for the bilin transition of longest wavelength (S1) as 15Z or 15E. Multiple values for the 15E state of NpTCCP reflect thermal evolution of the yellow-orange intermediate to a red-absorbing photoproduct (15).

γ Specific absorbance ratio (SAR) values of acid-urea denatured proteins were calculated as the ratio of the peak absorbance of the bilin band and the protein band at 280 nm, serving as a relative measure of chromophore-binding efficiency. For BV adducts, SAR values indicated with asterisks correspond to native proteins due to instability of the presumed non-covalent adducts under acid denaturing conditions.

δ Chromophorylation yield (Yc) was calculated as the ratio of bound chromophore bound and to protein content (see Materials and Methods for details).

ε Relative photoconversion efficiency of native BV- or ΦB-chromophorylated proteins (%ΔΔA) to respective PCB-chromophorylated proteins were provided. Photoconversion efficiency given in parenthesis was determined by subtracting the 15E spectrum from the 15Z spectrum. Spectra were normalized to the blue band absorbance prior to the calculation. Value in parenthesis is photoconversion efficiency.

na, not applicable.
Table 2. Spectral properties of PCB-adducts of ToTCCP wild type and variants.\textsuperscript{a}

| Protein     | $\lambda_{\text{max}}$ | SAR\textsuperscript{b} | $%\Delta A$ | Reverse photocycle | Remarks                        |
|-------------|------------------------|-------------------------|-------------|-------------------|--------------------------------|
| Wild type   | 402; 662               | 0.191                   | 100         | Y                 |                                |
| **Cys Variants** |                        |                          |             |                   |                                |
| C257A       | 402; 402               | 0.06                    | na          | N                 | Canonical 1\textsuperscript{st} Cys |
| C257S       | 398; 402               | 0.107                   | na          | N                 |                                |
| C258H       | 567; 627               | 0.114                   | na          | N                 | TP specific axial Cys          |
| C258S       | 647; 664               | 0.318                   | 100         | Y                 |                                |
| C258D       | 552; 672               | 0.027                   | 85.4        | Y                 |                                |
| C258I       | 566; 610               | 0.021                   | 67.7        | Y                 |                                |
| C258F       | 545; 545               | 0.111                   | na          | N                 |                                |
| C258Y       | 580; 580               | 0.010                   | na          | N                 |                                |
| C287S       | 403; 663               | 0.159                   | 94.5        | Y                 | Cph conserved Cys               |
| C303S       | 403; 663               | 0.179                   | 86.9        | Y                 | Cph conserved Cys               |
| **Other Variants** |                        |                          |             |                   |                                |
| H262L       | 427; 665               | 0.121                   | 98.1        | Y                 | TCCP signature                 |
| H262Y       | 403; 662               | 0.146                   | 80.4        | Y                 |                                |
| L270S       | 398; 662               | 0.231                   | 139.9       | Y                 |                                |
| V272T       | 402; 598, 604          | 0.454                   | 22.9, 35.9  | Y                 |                                |
| V272S       | 408; 578               | 0.235                   | 77.6        | Y                 | TCCP signature                 |
| S286A       | 396; 598, 662          | 0.186                   | 57.3, 84.9  | Y                 | TCCP signature                 |
| V254F       | 408; 660               | 0.122                   | 98.1        | Y                 | variant in NpTCCP              |
| A256W       | 407; 658               | 0.097                   | 75.7        | Y                 | variant in NpTCCP              |
| F261Y       | 404; 602               | 0.045                   | 70.4        | Y                 | variant in ToTCCP              |
| F261W       | 405; 602, 662          | 0.109                   | 43.6, 85.4  | Y                 |                                |
| G268K       | 411; 659               | 0.053                   | 101.6       | Y                 | variant in NpTCCP              |
| D205N       | 405; 597               | 0.088                   | 45.4        | Y                 |                                |
| Y174F       | 403, 578               | 1.04                    | 52          | Y                 |                                |
| Y174H       | 405, 565               | 0.81                    | 50.6        | Y                 |                                |
| H288A       | 382, 583               | 0.64                    | 21.9        | Y                 |                                |
| Y201F       | 403, 655               | 0.84                    | 75.7        | Y                 |                                |

\textsuperscript{a}Recombinant photosensory core domains (PAS-GAF-PHY) of ToTCCP were purified after expression in \textit{E. coli} cultures synthesizing PCB (from Figs. 4, 5) and dissolved in 50 mM Tris-HCl buffer (pH 8) containing 12 mM NaCl and 10 mM imidazole. Label abbreviations are defined in Table 1.

\textsuperscript{b}Specific Absorbance Ratio (SAR), a measure of chromophorylation, is the ratio of absorbances at the longest wavelength absorption maximum of the dark state and at 280 nm.

na, not applicable
Figure 1. Proposed TCCP photocycle and SyCph1 GAF domain structure labeled with chromophore-containing signature residues.

A. Proposed photocycle. TCCP dark states absorb violet-to-blue light due to two thioether linkages to the PCB chromophore (15). Light absorption by the $^{15Z}$Pvb ‘dark state’ triggers ultrafast isomerization of the $^{15Z}$ double bond of the doubly-linked PCB chromophore that destabilizes the thioether linkage at C10 to generate a YO-absorbing $^{15E}$Pyo ‘ion pair’ photoproduct with a re-aromatized chromophore. The $^{15E}$Pyo photoproduct equilibrates with a R-absorbing $^{15E}$Pr photoproduct – a process requiring D205 and favored at low pH (as shown by this investigation). We envisage this process to entail a repositioning of the D-ring to interact with the D205 carboxylate, likely in conjunction with rearrangement of the tongue region of the adjacent PHY domain seen in other phytochromes (30, 31, 48). This interconversion can be very fast (< sec) or quite slow (>min, as seen for NpTCCP) depending on TCCP species or variant. The $^{15E}$Pyo/$^{15E}$Pr equilibrium also varies amongst TCCP family members and can be altered by site-directed mutations of conserved TCCP-signature residues in both GAF and PHY domains (as shown by this investigation). Both $^{15E}$Pyo and $^{15E}$Pr photoproduct states are photoactive since YO or R light can trigger regeneration of the $^{15Z}$Pvb dark state. Residues in ToTCCP which likely interact with the chromophore are shown in parentheses.

B. Chromophore-contacting signature residues of SyCph1 and corresponding residues found in ToTCCP. Shown is the chromophore-binding pocket of the SyCph1 GAF domain with PCB in the R-absorbing Pr state (PDB ID 2VEA) (28). The chromophore-binding pocket is drawn in surface representation (light grey). Labels refer to amino acid sequence numbers, and PCB is colored in teal and shown in stick form. Key chromophore-contacting residues are labeled with black font for SyCph1. ToTCCP residues conserved with or diverged from those in Cph1 are labeled (in brackets) with red or blue fonts, respectively.
Figure 2. Phylogenetic analysis of photosensory core modules of TCCP, Cph1/CphA and BphP/CphB family representatives supports a monophyletic TCCP lineage in cyanobacteria. The TCCP family consisting of NpTCCP (NpF1183) from Nostoc punctiforme ATCC 29133 (15), orthologues ToTCCP from Tolypothrix PCC7910 (Tpr0787), ScTCCP from Scytonema PCC10023 (Spr6432) and GpTCCP from Gloeocapsa PCC7513 (Gpr2095) studied here (indicated with arrows), comprise a monophyletic clade distinct from Cph1/CphA and BphP/CphB cyanobacterial phytochrome families. Non-cyanobacterial BphPs are included as an outgroup. Detailed sequence names and accessions are provided in Supplemental Material (Table S2). Sequence alignments are included as a Supplementary datafile.
Figure 3. Spectral properties of bilin adducts of PCMs of recombinant ToTCCP, ScTCCP and GlTCCP proteins indicate differences in the ratio of two spectrally distinct 15E photoproducts in the TCCP family. Absorption spectra of PCMs of ToTCCP (A), ScTCCP (B), and GlTCCP (C) incorporating PCB (solid line), BV (dotted line), or PΦB (dashed line) are shown in 15Z dark states (blue) and 15E photoproducts (orange) in standard assay buffer at pH 8 (see Materials and Methods). Dark-minus-light difference spectra at pH 8 for native ToTCCP (D), ScTCCP (E), and GlTCCP (F) obtained from panels A, B, and C, respectively. Zinc in-gel fluorescence and Coomassie Brilliant Blue (CBB) images of acid-denatured TCCPs (G). SDS-PAGE gels imaged by zinc-dependent in-gel fluorescence (upper panel) and after staining with CBB (lower panel). Molecular weight markers (M) in kDa are indicated on the ordinate on the right side.
Figure 4. Absorption spectra of Cys variants of ToTCCP indicates an essential role for the TCCP-signature cysteine in forming the B-absorbing 15Z dark state. Absorption spectra of PCMs of ToTCCP, canonical Cys variants, C257A (A) and C257S (B), and TCCP-signature cysteine variants, C258H (C), C258S (D), C258D (E), C258I (F), C258F (G) and C258Y (H), at pH 8 incorporating PCB are shown for 15Z dark states (blue) and 15E photoproducts (orange). The loss of the canonical Cys in C257A/S variants yields a B-absorbing 15Z species that affords a stable B-absorbing 15E state owing to the apparent stability of the C10 linkage. The loss of the TCCP-signature Cys (C258H/S/D/I/F/Y) yields red-absorbing variants in both 15Z and 15E states due to the loss of the C10 thioether linkage.
Figure 5. Absorption spectra of ToTCCP variants in TCCP family-specific, ToTCCP-specific and TCCP-family variable residues reveal selective roles in spectral tuning in the TCCP family. 15Z dark states shown in blue, 15E photoproduction states shown in orange before and after dark maturation (solid and dashed, respectively). Zinc in-gel fluorescence data for all mutants are shown in Figure S3.
Figure 6. Spectroscopic pH titrations of ToTCCP and its singly-linked C258S variant reveals pH-dependent equilibria between G- and R-absorbing species. ToTCCP wildtype and C258S variant in 15Z dark and 15E lit states at pH 8 were adjusted to different pHs (See Materials and Methods for details). All spectra are normalized to the same concentration in panels A, B, D and E. In panel C and F, the 15E photoproduct at pH 7 and pH 10 (solid curves, blue pH 10 and orange pH 7) were adjusted to pH 7 or pH 10 (dashed curves, blue pH 7 and orange pH 10). Normalized spectra shown are superimposed.
Figure 7. pH dependence of selected variants of ToTCCP indicates that the 15E photoproduct comprises a mixture of spectrally distinct YO-absorbing 'deprotonated' and R-absorbing 'protonated' states. ToTCCP variants after conversion to their 15E photoproduct states were adjusted to different pHs (see Materials and Methods for details). Spectra for each indicated variant are colored by pH as follows: pH 6 (red), pH 8 (green) and pH 11 (fushia).
Spectral and photochemical diversity of tandem cysteine cyanobacterial phytochromes
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