Published in final edited form as:

Curr Opin Struct Biol. 2019 March 14; 57: 72–83. doi:10.1016/j.sbi.2019.02.005.

Bacteriophytochromes - from informative model systems of phytochrome function to powerful tools in cell biology

Geoffrey Gourinchas^{#1}, Stefan Etzl^{#1}, and Andreas Winkler^{1,2,*}

¹Institute of Biochemistry, Graz University of Technology, Petersgasse 12/II, 8010 Graz, Austria

²BioTechMed-Graz, 8010 Graz, Austria

[#] These authors contributed equally to this work.

Abstract

Bacteriophytochromes are a subfamily of the diverse light responsive phytochrome photoreceptors. Based on their preferential interaction with biliverdin IX α as endogenous cofactor, they have recently been used for creating optogenetic tools and engineering fluorescent probes. Ideal absorption characteristics for activation of bacteriophytochrome-based systems in the therapeutic near-infrared window as well the availability of biliverdin in mammalian tissues have resulted in tremendous progress in re-engineering bacteriophytochromes for diverse applications. At the same time, the structural and functional characterization of diverse naturally occurring bacteriophytochrome systems has unraveled remarkable differences in signaling mechanisms and has so far only touched the surface of the evolutionary diversity within the family of bacteriophytochromes. This review highlights recent findings and future challenges.

Introduction

Phytochromes are photoreceptor proteins employed by bacteria, fungi, algae and plants to sense ambient light quality and quantity. Historically, they were annotated as red/far-red light sensors, however, the variability in domain organization (Figure 1a), different photochemistries of the light absorbing bilin cofactors [1] and amino acid variability near the cofactor [2,3] enable tuning of the spectral properties within the whole ultra-violet, visible and near-infrared (NIR) regions of the electromagnetic spectrum. From an evolutionary perspective, phytochromes have originated in the bacterial kingdom and extensively diversified in different phyla over billions of years with frequent gene loss and horizontal transfers, complicating phylogenetic analyses of the phytochrome sensory module [4]. The relationship and functional correlation of full-length phytochrome-effector combinations is additionally complicated by the diversity of output domains that are frequently linked to the photoreceptor C-termini (Figure 1a). In that respect, this review focuses on recent progress made in understanding and exploiting the modularity within the family of bacteriophytochromes, which serve as powerful model systems for the more complex plant phytochromes [5] and have attracted special attention for optogenetic tool development as well as engineering of fluorescent reporters.

Corresponding author: Andreas.Winkler@TUGraz.at.

Sensor-effector modularity in bacteriophytochromes

One central question with respect to bacteriophytochrome function is tightly linked to the observed modularity of output functionalities (Figure 1a): “Is there a common molecular mechanism for phytochrome activation and regulation of diverse enzymatic functionalities or protein-protein interactions?” As described in detail below, light absorption by the biliverdin IX α (BV) chromophore (Figure 1b and 1c) results in complex structural rearrangements affecting several structural elements of phytochrome sensors. Therefore, evolutionary processes have likely selected alternative signaling routes for adaption to specific effector requirements such as control of protein-protein interactions [6] or regulation of enzymatic activities [7,8]. Consequently, reengineering of bacteriophytochrome sensors holds great potential for regulating non-natural output functionalities [9] and converting red light sensors to fluorescent reporters [10]. However, a rational approach to design novel sensor-effector combinations remains challenging and requires a more detailed understanding of the diversity in signaling mechanisms of full-length natural systems.

To this end, research has focused on identifying molecular mechanisms involved in long-range signal transduction from the site of chromophore activation to, for example, the active site of downstream enzymatic effectors. Considering the importance of protein dynamics, the multi-domain organization and the dimeric nature of bacteriophytochromes (Figure 2a), obtaining structural information of full-length systems in multiple functionally relevant states is challenging. Therefore, many studies have initially focused on the isolated photosensory modules (PSM) to unravel structural details of phytochrome signaling and how structural changes correlate with the typical spectroscopic signatures of phytochrome absorbing in the red and far-red region (Pr and Pfr states, respectively; Figure 1b).

Functionally important phytochrome elements

Photosensory modules of bacteriophytochromes are composed of Period/ARNT/Single-minded (PAS), cGMP phosphodiesterase/adenylyl cyclase/FhlA (GAF), and Phytochrome-specific (PHY) domains (Figure 1a). Even though the PAS and PHY domains have frequently been lost during evolution and GAF-only constructs can operate as stand-alone photosensors [11], both accessory domains play important roles in specific aspects of bacteriophytochrome signaling.

In fact, short sequences extending from the PAS N-termini – the N-terminal segment (NTS, Figure 2b,c,d) – not only provide the conserved cysteine involved in covalent adduct formation with the C3² atom of BV (Figure 1c and 2b) but also constitute an essential part of the characteristic phytochrome knot architecture [12] (Figure 2e). In contrast to the longer N-terminal extensions (NTE) of plant phytochromes that play important roles in downstream signaling [13] and chromophore integrity [14], the bacteriophytochrome NTS is poorly understood with respect to its involvement in Pr to Pfr transitions. A detailed comparison of structural data, however, indicates a direct correlation of the NTS and the characteristic PHY-tongue element with the BV configuration (Figure 2c) [7,15–21]. In fact, characteristics of the cysteine-linkage of BV and the A-ring geometry correlate with the NTS placement (Figure 2b). Consequently, the NTS serves as an additional modulator of

bacteriophytochrome signaling via its molecular cross-talk with elements from the PHY domain [22]. It is tempting to speculate that the characteristic knot structure that reduces the degrees of freedom in the PAS-GAF interaction [12] also favors the efficient interaction of the NTS and the PHY-tongue close to the BV pocket, which is also present in cyanobacterial Cph1 lacking the NTS-cysteine for bilin tethering [23].

As indicated above, the C-terminal PHY domain serves as second accessory PSM module; on the one hand, by connecting its C-terminal helix to linker elements involved in effector regulation [7,16,24–26] and, on the other hand, by providing the characteristic PHY-tongue element that was shown to be a central structural element for signal propagation [15,24,27,28]. Notably, the characteristic differences in tongue conformation between bathyphytochromes, featuring the Pfr-state as dark-adapted conformation [6,29,30], and canonical bacteriophytochromes in Pr [7,18,19,21] early on indicated how local rearrangements in the BV binding pocket are linked to changes in PHY-tongue secondary structure. It is well established that the transition between a β -hairpin conformation in Pr and an α -helical conformation in Pfr (Figure 2c) is one central element of signal transduction in phytochromes and that specific regions, such as the DIP and PRXSF motifs (located close to BV in the GAF domain and the PHY-tongue, respectively; Supplementary Figure 1) serve as relay between tongue rearrangements and reorganization of the BV environment [1].

Connecting the biliverdin environment with bacteriophytochrome function

The central element of the PSM is the GAF domain as it provides the BV binding pocket, which is lined by many highly conserved residues (Figure 2d, Supplementary Figure 1). BV adopts a $5Z_{syn}, 10Z_{syn}, 15Z_{anti}$ conformation (ZZZ_{ssa} or $15Z$) in the Pr state and is typically covalently linked to a conserved cysteine of the NTS by 1,4 addition to the vinyl group of ring A in bacteriophytochromes (Figure 1c). As indicated above, the NTS and the PHY-tongue also shield the cofactor from solvent exposure [12,15,18–21,31,32] and appropriately position the cofactor for BV isomerization at its C15-C16 double bond upon light activation. This isomerization reaction results in D-ring rotation and eventually establishes the characteristic $5Z_{syn}, 10Z_{syn}, 15E_{anti}$ conformation (ZZE_{ssa} or $15E$) of the Pfr-state, as observed in bathyphytochrome structures [6,29,30], but recently also for activated canonical bacteriophytochromes [15,24,33]. Due to the extensive BV binding pocket, isomerization and rotation at the C-D methine bridge depend on steric properties of the chromophore environment [34]. Interestingly, the covalent BV-linkage is not essential for Pr to Pfr photoconversion and has no effect on signal transduction to the effector [35]. However, studies based on the PAS-GAF core of *Deinococcus radiodurans* DrBphP showed that the position of the cysteine involved in adduct formation (either NTS or GAF) has a strong impact on spectral properties [36].

In line with studies indicating conformational changes upon BV isomerization [37–39], differences between Pr and Pfr structures highlighted characteristic rotamer changes of residues surrounding the BV D-ring (Figure 2d). Notably, Tyr168 (*IsPadC* numbering) is highly conserved and coupled to D-ring rotation [7,15,24]. Also, His193 and Phe195 (*IsPadC* numbering; cf. Supplementary Figure 1), albeit not strictly conserved, are linked to D-ring rotation. The position of His193 appears interesting since it has a strong influence on

the stability of 15EBV [30,40], but is quite variable in that some bacteriophytochromes feature Leu and, frequently bathyphytochromes, Asn or Gln at the same position. Importantly, replacement of Gln by a hydrophobic residue in *Pseudomonas aeruginosa* PaBphP resulted in a significantly reduced Pfr-state stability [29].

As far as structural intermediates along the route from Pr to Pfr or vice versa are concerned, it is generally accepted that rapidly formed Lumi intermediates (Lumi-R in the Pr to Pfr conversion and Lumi-F for Pfr to Pr) correspond to rotated D-ring conformations destabilizing characteristic interactions in the respective dark-adapted states [29,41]. The subsequently formed Meta-R or Meta-F intermediates feature reduced D-ring dynamics and a reorganization of residues surrounding BV resembling the respective end-states of Pfr and Pr, respectively [41,42]. In the last step of photoconversion, the characteristic Pfr and Pr states are formed by reorganization of the tongue and the NTS [24]. A recent Lumi-R model proposed that D-ring rotation destabilizes the highly conserved Asp207:Arg466 (*DrBphP* numbering) salt bridge between the DIP and PRXS motifs, by establishing altered hydrogen-bonding interactions for these two residues [41]. Upon Meta-R formation, interactions of the D-ring carbonyl are completely disrupted and eventually destabilize the β -hairpin conformation of the PHY-tongue to enable partial refolding to an α -helix upon Pfr formation [41,43].

Importantly, an atomic characterization of specific intermediates is complicated by the unpredictable influence of crystal lattice restraints on functionally important elements and the cross-talk between different bacteriophytochrome elements with variable degrees of uncoupling [44]. Additionally, the dimer interface of phytochromes, as discussed in the **Quaternary Structure** section (Figure 2f), and non-uniform reaction pathways including off-pathway shunt reactions [45,46] also contribute to heterogeneity. These caveats for obtaining homogeneous photoactivated preparations are further complicated by thermal recovery processes between Pfr- and Pr-states with pronounced differences in mean lifetimes. Nevertheless, several structural approaches have established a unifying concept of initial steps in signal integration by bacteriophytochromes [15,29,30,33]. As far as full-length canonical bacteriophytochromes are concerned, refolding of the PHY-tongue by destabilization of its Pr conformation, appears to be a conserved structural feature upon transition to Pfr that is eventually required to modulate the conformational dynamics of the PHY dimer interface and to transduce the light signal to downstream effectors (Figure 3) [15,16,24,47].

Sensor-effector coupling in bacteriophytochromes

Refolding of the PHY-tongue observed in *DrBphP* PSM crystal structures, revealed a pronounced effect on the PHY dimer interface [15,33] (Figure 3a). Subsequently, integrative models of full-length *DrBphP* coupled to its histidine kinase (HK) by means of time-resolved x-ray scattering [47] and cryo-EM [21,48] refined the displacement of the PHY domains and associated their bending motion to a rotation of the coiled-coil linker element and the HK effector (Figure 3a). Interestingly, an integrative structural biology approach for the PSM of *DrBphP* coupled to an artificial adenylyl/guanylyl cyclase effector suggested unfolding of the linker region upon red light activation resulting in release of an inhibited

effector conformation [16] (Figure 3). Such diverse mechanisms of sensor-linker-effector coupling triggered by the same bacteriophytochrome activation mechanism highlight the fine-tuned coupling of bacteriophytochromes and their cognate effectors, which is realized without pronounced global structural rearrangements as also suggested for Agp1 containing an HK output[49].

Moreover, recent structures of two naturally occurring systems showed even more diversity in sensor-linker-effector modulation [7,17]. Both full-length bacteriophytochromes from *Idiomarina sp. A28L* (*IsPadC*, phytochrome-activated diguanylate cyclase with GGDEF domain output) and *Xanthomonas campestris* (*XccBphP*, PAS-linked phytochrome) showed an increase in conformational dynamics at their helical sensor-effector linkers upon illumination. Based on structural comparison with the Pfr protomer of *Rhodospseudomonas palustris* (*RpBphP1*) [6], a hinge-like motion of the sensor-effector linker was suggested to induce a rotational movement of the PAS effector in *XccBphP* to modulate the interaction with unknown effectors [17]. Moreover, the comparison of homodimeric inhibited Pr-state crystal structures of *IsPadC* with an activated conformation featuring a Pr-Pfr heterodimer revealed a subtle hinge-like motion of one protomer that enables switching between two different conformational registers of the coiled-coil linker region and thereby increases diguanylyl cyclase activity [7,24] (Figure 3a,b). In fact, a similar type of Meta-R and Pfr asymmetry is also reflected in the characteristic spectral behavior of *IsPadC* (Figure 1b), which resembles observations for other bacteriophytochromes [20,23,50]. Therefore, asymmetric activation of bacteriophytochromes might be important for the regulation of specific effectors that feature asymmetric intermediates in their catalytic mechanisms [51] and, hence, also for the family of phytochrome linked HKs [52].

The role of the quaternary phytochrome structure

Most likely, the characteristics of asymmetry are modulated by the dimer interface, since the predominantly parallel phytochrome assemblies do not involve conserved interface residues and, therefore, apparently serve as evolutionary playground for tuning properties of the chromophore and/or sensor-effector coupling. Although parallel homodimers are observed for most full-length systems (Figure 3a), some truncated bacteriophytochrome species feature anti-parallel dimers upon crystallization [6,19,31]. This supports the flexibility of the dimer interfaces and anti-parallel dimers might potentially exist as inhibitory conformations or might be involved in systems regulating protein-protein interactions [6].

Based on full-length structures of bacteriophytochromes, the driving forces for dimer association have been attributed to the PAS-GAF interface [53], the sensor-effector linker region [7,16], or the effector domain depending on the output function [7,16,17]. Notably, the PSM of *DrBphP* could be monomerized by reducing only the GAF interface stability, highlighting the importance of these interactions [53]. Nevertheless, the flexibility of PSM interfaces, reflected in different geometries of the central helical spine connecting the PAS-GAF core to the PHY domain (Figure 2f), might influence the contribution of the PHY domain interface to the overall dimer stability. As a result, the PHY interface, which is tightly linked to the conformation of the PHY-tongue, appears as an additional regulator for signal specificity and downstream communication (Figure 3a).

The role of the PAS-GAF and PHY interfaces in quaternary structure organization and occurrence of asymmetry is supported by observations of typically symmetrically activated *DrBphP* [21], where engineered PAS-GAF fragments showed allosteric communication of the two protomers as reflected in heterogeneous chromophore environments [36]. Similarly, monomerization of the *DrBphP* PAS-GAF construct reestablishes photoconversion, which is otherwise inhibited by the dimer interface [54] and, even in the context of monomerized PSM constructs, it was shown that the dimer interface is in principle dispensable for photoconversion [53]. However, more directed experiments addressing asymmetry in bacteriophytochromes are required to fully appreciate the complex interplay of structural elements of the PSM dimer and its cross-talk with the sensor-effector linker. Importantly, the frequently observed chromophore heterogeneity [24,30,46] might be influenced by allosteric communication between the protomers and the highly flexible phytochrome dimer interfaces [7,31].

Engineering bacteriophytochromes with improved fluorescent properties

While phytochrome dimerization and the presence of accessory PAS and PHY modules are important for functioning as light sensors, smaller constructs with decreased quantum yields for BV isomerization are generally desired for exploiting the weak fluorescence of bacteriophytochromes. BV is readily available in higher eukaryotic organisms as a product of heme catabolism and bacteriophytochromes exhibit the most red-shifted spectra within the deep-penetration-window of NIR-light, rendering bacteriophytochrome-based systems advantageous for optogenetic applications in mammalian systems.

The development of NIR fluorescent proteins (NIR-FPs) generally attempts to destabilize photocycle intermediates with the aim of increasing radiative decay of the excited state to enhance fluorescence quantum yields. This is usually realized by utilization of the chromophore binding domains (CBD, PAS-GAF) of BphPs and amino acid substitutions around the chromophore (e.g. targeting Asp or Ile of the conserved DIP motif or Y263 according to *DrBphP* numbering [44,54]). Eventually, quantum yields of BphP-derived FPs can reach up to 14% [55]. Initial NIR-FPs were based on Cph1 (PR-1) and *DrBphP* (IFP1.4, IFP2.0, IFP1.4rev, Wi-Phy) (summarized in Chernov *et al.* 2017 [10]). Early systems suffered from inefficient BV incorporation or the requirement for bilins not present in mammalian cells. To this end, other systems based on *RpBphP2* (iRFP [10]) or *Nostoc* AphB [56] were developed with higher binding efficiencies of endogenous BV or optimized spectral properties by using *RpBphP2* and *RpBphP6* [10]. Despite their applicability for whole-cell imaging, dimeric NIR-FPs still show drawbacks in their application as protein fusion tags, because their oligomeric state might affect the functionality of target proteins (Figure 4a).

Due to this limitation, monomeric NIR fluorescent proteins truncated to the CBD have been developed [6,57]. BphP1-FP and several miRFPs are based on *RpBphP1* PAS-GAF [55] and mIFP and its derivative iBlueberry have been engineered from *Bradyrhizobium* *BrBphP* [57,58]. In order to study protein-protein interactions using bimolecular fluorescence complementation, several split versions of IFP [59,60] and iRFPs [61,62] have also been engineered (Figure 4b). Notable exceptions from the typical CBD architecture are PAiRFPs

and GAF-FP. PAiRFPs feature engineered variants of the whole PSM of the bathyphytochrome *AfAgp2* and thereby provide NIR-activatable fluorescent systems [63]. Conversely, GAF-FP is a small single domain FP featuring an engineered, knot-less monomeric GAF domain from *RpBphP1* [64] (Figure 4a).

Recent applications using non-fluorescent phytochromes include advanced imaging techniques using photoacoustic tomography and photoacoustic microscopy. In this context, the usability of *RpBphP1* was demonstrated [65] and the concept further improved with utilization of the *DrBphP* PSM for imaging and the development of its split variant, *DrSplit*, for detecting protein-protein interactions [66] (Figure 4b). An exhaustive overview of fluorescent proteins and development strategies based on BphPs is provided in Chernov et al. [10]. Interestingly, substitutions of specific amino acids in the BV environments close to the D-ring result in similar effects on fluorescence quantum yields [10,67] (Figure 4), however, random mutagenesis efforts frequently also provide unpredictable variants with beneficial properties in different bacteriophytochrome backgrounds, further supporting the notion of subtle differences in phytochrome mechanisms.

Engineering bacteriophytochrome-based optogenetic tools

While several studies report the successful generation of fluorescent reporters by reducing the complexity of bacteriophytochromes, re-engineering BphPs to regulate novel enzymatic output functionalities is more challenging. Nevertheless, engineered BphPs have been exploited as red/far-red light dependent switches to regulate non-natural enzymatic effectors (Figure 4c), including cyclases (IlaC [26], PagC/PaaC [16]) and phosphodiesterases (LAPD [25]). Naturally occurring diguanylate cyclase functionalities (with exchanged output domain in BphS [68] compared to *RsBphP1*) have recently also been applied for NIR light-controllable biofilm formation [69]. While the importance of finding the correct fusion points and sequence composition of helical sensor-effector linkers is common to all these engineering approaches, the molecular basis for how the signal is transduced is frequently not understood in detail. In fact, single amino acid replacements at seemingly unimportant positions in the linker can have a profound impact on dynamic ranges and functionality [16,25,68].

Additionally, utilizing the capability of red light-regulated homo- or heterodimerization of either Cph1 [70] or the bathyphytochrome *RpBphP1* and its interaction partner *RpPpsR2* [71], respectively, was exploited to control intracellular targeting and relocalization of proteins. Improved versions of the latter system were recently developed by reducing the PpsR2 complexity to subdomains Q-PAS1 [72], as well as by generating chimeras of Q-PAS1 and *AsLOV2* which allow tri-directional protein targeting [73] (Figure 4d).

Conclusions

While a rational design of tools derived from bacteriophytochromes is still challenging and most engineered systems involve substantial random mutagenesis and directed evolution approaches, the functional characterization of some of the resulting proteins also frequently provides new insights into the molecular mechanisms of phytochrome function and

signaling. With more bacteriophytochrome systems being characterized, the near future will provide a more complete picture of bacteriophytochrome function, highlighting many conserved features. At the same time, it is likely that evolution has created different subtypes of phytochrome function involving symmetric and asymmetric properties. Understanding and exploiting such peculiarities will open an even broader toolbox for future applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

G.G. and S.E. are supported by the by the Austrian Science Fund through the PhD program “DK Molecular Enzymology” (W901). The authors gratefully acknowledge support from NAWI Graz. A.W. acknowledges funding by the Austrian Science Fund (FWF): P27124.

References

- Anders K, Essen L-O. The family of phytochrome-like photoreceptors: diverse, complex and multi-colored, but very useful. *Curr Opin Struct Biol.* 2015; 35:7–16. [PubMed: 26241319]
- Rockwell NC, Martin SS, Feoktistova K, Lagarias JC. Diverse two-cysteine photocycles in phytochromes and cyanobacteriochromes. *Proc Natl Acad Sci.* 2011; 108:11854–11859. [PubMed: 21712441]
- Rockwell NC, Duanmu D, Martin SS, Bachy C, Price DC, Bhattacharya D, Worden AZ, Lagarias JC. Eukaryotic algal phytochromes span the visible spectrum. *Proc Natl Acad Sci.* 2014; 111:3871–3876. [PubMed: 24567382]
- Rockwell NC, Lagarias JC. Phytochrome diversification in cyanobacteria and eukaryotic algae. *Curr Opin Plant Biol.* 2017; 37:87–93. [PubMed: 28445833]
- Burgie ES, Vierstra RD. Phytochromes: An Atomic Perspective on Photoactivation and Signaling. *Plant Cell Online.* 2014; 26:4568–4583.
- Bellini D, Papiz MZ. Structure of a Bacteriophytochrome and Light-Stimulated Protomer Swapping with a Gene Repressor. *Structure.* 2012; 20:1436–1446. [PubMed: 22795083]
- Gourinchas G, Etzl S, Göbl C, Vide U, Madl T, Winkler A. Long-range allosteric signaling in red light-regulated diguanylyl cyclases. *Sci Adv.* 2017; 3:e1602498. [PubMed: 28275738]
- Baker AW, Satyshur KA, Moreno Morales N, Forest KT. Arm-in-Arm Response Regulator Dimers Promote Intermolecular Signal Transduction. *J Bacteriol.* 2016; 198:1218–1229. [PubMed: 26833410]
- Ziegler T, Möglich A. Photoreceptor engineering. *Front Mol Biosci.* 2015; 2
- Chernov KG, Redchuk TA, Omelina ES, Verkhusha VV. Near-Infrared Fluorescent Proteins, Biosensors, and Optogenetic Tools Engineered from Phytochromes. *Chem Rev.* 2017; 117:6423–6446. [PubMed: 28401765]
- Narikawa. tentative title - Natural and engineered cyanobacteriochromes. *Curr Opin Struct Biol.* 2019
- Wagner JR, Brunzelle JS, Forest KT, Vierstra RD. A light-sensing knot revealed by the structure of the chromophore-binding domain of phytochrome. *Nature.* 2005; 438:325–331. [PubMed: 16292304]
- Burgie ES, Bussell AN, Walker JM, Dubiel K, Vierstra RD. Crystal structure of the photosensing module from a red/far-red light-absorbing plant phytochrome. *Proc Natl Acad Sci.* 2014; 111:10179–10184. [PubMed: 24982198]
- Velázquez Escobar F, Buhrke D, Fernandez Lopez M, Shenkutie SM, von Horsten S, Essen L-O, Hughes J, Hildebrandt P. Structural communication between the chromophore-binding pocket and the N-terminal extension in plant phytochrome phyB. *FEBS Lett.* 2017; 591:1258–1265. [PubMed: 28376244]

15. Takala H, Björling A, Berntsson O, Lehtivuori H, Niebling S, Hoernke M, Kosheleva I, Henning R, Menzel A, Ihalaainen JA, et al. Signal amplification and transduction in phytochrome photosensors. *Nature*. 2014; 509:245–248. [PubMed: 24776794]
16. Etzl S, Lindner R, Nelson MD, Winkler A. Structure-guided design and functional characterization of an artificial red light-regulated guanylate/adenylate cyclase for optogenetic applications. *J Biol Chem*. 2018; doi: 10.1074/jbc.RA118.003069 [• Consideration of an important regulatory element of adenylate/guanylate cyclases enabled the generation of improved optogenetic tools with reduced dark state activities and increased dynamic ranges. Application in nematodes demonstrated the *in vivo* functionality of these artificial phytochrome activated nucleotide cyclases.]
17. Otero LH, Klinker S, Rinaldi J, Velázquez-Escobar F, Mroginski MA, Fernández López M, Malamud F, Vojnov AA, Hildebrandt P, Goldbaum FA, et al. Structure of the Full-Length Bacteriophytochrome from the Plant Pathogen *Xanthomonas campestris* Provides Clues to its Long-Range Signaling Mechanism. *J Mol Biol*. 2016; 428:3702–3720. [PubMed: 27107635] [• Description of the full-length structure of a bacteriophytochrome coupled to a non-enzymatic effector domain. Biochemical data supported the importance of the sensor-effector linker region and the output module on the phytochrome photo-conversion and highlighted the bidirectional allosteric communication between output domains and phytochrome sensors]
18. Woitowich NC, Halavaty AS, Waltz P, Kupitz C, Valera J, Tracy G, Gallagher KD, Claesson E, Nakane T, Pandey S, et al. Structural basis for light control of cell development revealed by crystal structures of a myxobacterial phytochrome. *IUCrJ*. 2018; 5:619–634.
19. Nagano S, Scheerer P, Zubow K, Michael N, Inomata K, Lamparter T, Krauß N. The Crystal Structures of the N-terminal Photosensory Core Module of *Agrobacterium* Phytochrome Agp1 as Parallel and Anti-parallel Dimers. *J Biol Chem*. 2016; 291:20674–20691. [PubMed: 27466363]
20. Yang X, Stojković EA, Kuk J, Moffat K. Crystal structure of the chromophore binding domain of an unusual bacteriophytochrome, RpBphP3, reveals residues that modulate photoconversion. *Proc Natl Acad Sci*. 2007; 104:12571–12576. [PubMed: 17640891]
21. Burgie ES, Wang T, Bussell AN, Walker JM, Li H, Vierstra RD. Crystallographic and Electron Microscopic Analyses of a Bacterial Phytochrome Reveal Local and Global Rearrangements during Photoconversion. *J Biol Chem*. 2014; 289:24573–24587. [PubMed: 25006244]
22. Gourinchas G, Vide U, Winkler A. Influence of the N-terminal segment and the PHY-tongue element on light-regulation in bacteriophytochromes. *J Biol Chem*. 2019; doi: 10.1074/jbc.RA118.007260
23. Essen L-O, Mailliet J, Hughes J. The structure of a complete phytochrome sensory module in the Pr ground state. *Proc Natl Acad Sci*. 2008; 105:14709–14714. [PubMed: 18799745]
24. Gourinchas G, Heintz U, Winkler A. Asymmetric activation mechanism of a homodimeric red light-regulated photoreceptor. *eLife*. 2018; 7:e34815. [PubMed: 29869984] [•• This work reported the three-dimensional structure of a full-length phytochrome regulated diguanylate cyclase in an activated conformation, supporting the involvement of the coiled-coil sensor-effector linker in signal transduction and regulation of the enzymatic effector. The coiled-coil linker can adopt two different conformations, which are coupled to structural rearrangements of the phytochrome tongue in an asymmetric phytochrome dimer. In comparison to other activated bacteriophytochrome structures, the study suggested that phytochromes might have evolved different long-range signal transduction mechanisms.]
25. Gasser C, Taiber S, Yeh C-M, Wittig CH, Hegemann P, Ryu S, Wunder F, Möglich A. Engineering of a red-light-activated human cAMP/cGMP-specific phosphodiesterase. *Proc Natl Acad Sci*. 2014; 111:8803–8808. [PubMed: 24889611]
26. Ryu M-H, Kang I-H, Nelson MD, Jensen TM, Lyuksyutova AI, Siltberg-Liberles J, Raizen DM, Gomelsky M. Engineering adenylate cyclases regulated by near-infrared window light. *Proc Natl Acad Sci*. 2014; 111:10167–10172. [PubMed: 24982160]
27. Anders K, Gutt A, Gärtner W, Essen L-O. Phototransformation of the Red Light Sensor Cyanobacterial Phytochrome 2 from *Synechocystis* Species Depends on Its Tongue Motifs. *J Biol Chem*. 2014; 289:25590–25600. [PubMed: 25012656]
28. Song C, Mroginski MA, Lang C, Kopycki J, Gärtner W, Matysik J, Hughes J. 3D Structures of Plant Phytochrome A as Pr and Pfr From Solid-State NMR: Implications for Molecular Function. *Front Plant Sci*. 2018; 9

29. Yang X, Kuk J, Moffat K. Conformational differences between the Pfr and Pr states in *Pseudomonas aeruginosa* bacteriophytochrome. *Proc Natl Acad Sci.* 2009; 106:15639–15644. [PubMed: 19720999]
30. Yang X, Kuk J, Moffat K. Crystal structure of *Pseudomonas aeruginosa* bacteriophytochrome: Photoconversion and signal transduction. *Proc Natl Acad Sci.* 2008; 105:14715–14720. [PubMed: 18799746]
31. Yang X, Stojkovi EA, Ozarowski WB, Kuk J, Davydova E, Moffat K. Light Signaling Mechanism of Two Tandem Bacteriophytochromes. *Structure.* 2015; 23:1179–1189. [PubMed: 26095026]
32. Wagner JR, Zhang J, Brunzelle JS, Vierstra RD, Forest KT. High Resolution Structure of *Deinococcus* Bacteriophytochrome Yields New Insights into Phytochrome Architecture and Evolution. *J Biol Chem.* 2007; 282:12298–12309. [PubMed: 17322301]
33. Burgie ES, Zhang J, Vierstra RD. Crystal Structure of *Deinococcus* Phytochrome in the Photoactivated State Reveals a Cascade of Structural Rearrangements during Photoconversion. *Structure.* 2016; 24:448–457. [PubMed: 26853942] [• Description of the three-dimensional structure of the nearly completely light activated photosensory module of *DtBphP*. Symmetric activation of the bacteriophytochrome homodimer was supported by both chromophore environments featuring the *ZZEssa* biliverdin configuration and characteristic rotamer conformations of specific residues in the vicinity of the rotated D-ring.]
34. Falklöf O, Durbeej B. Steric Effects Govern the Photoactivation of Phytochromes. *ChemPhysChem.* 2016; 17:954–957. [PubMed: 26756452]
35. Borucki B, Seibeck S, Heyn MP, Lamparter T. Characterization of the Covalent and Noncovalent Adducts of Agp1 Phytochrome Assembled with Biliverdin and Phycocyanobilin by Circular Dichroism and Flash Photolysis. *Biochemistry.* 2009; 48:6305–6317. [PubMed: 19496558]
36. Stepanenko OV, Baloban M, Bublikov GS, Shcherbakova DM, Stepanenko OV, Turoverov KK, Kuznetsova IM, Verkhusha VV. Allosteric effects of chromophore interaction with dimeric near-infrared fluorescent proteins engineered from bacterial phytochromes. *Sci Rep.* 2016; 6
37. Inomata K, Khawn H, Chen L-Y, Kinoshita H, Zienicke B, Molina I, Lamparter T. Assembly of *Agrobacterium* Phytochromes Agp1 and Agp2 with Doubly Locked Bilin Chromophores †. *Biochemistry.* 2009; 48:2817–2827. [PubMed: 19253981]
38. Song C, Psakis G, Lang C, Mailliet J, Gärtner W, Hughes J, Matysik J. Two ground state isoforms and a chromophore D-ring photoflip triggering extensive intramolecular changes in a canonical phytochrome. *Proc Natl Acad Sci.* 2011; 108:3842–3847. [PubMed: 21325055]
39. Yang X, Ren Z, Kuk J, Moffat K. Temperature-scan cryocrystallography reveals reaction intermediates in bacteriophytochrome. *Nature.* 2011; 479:428–432. [PubMed: 22002602]
40. Zienicke B, Molina I, Glenz R, Singer P, Ehmer D, Escobar FV, Hildebrandt P, Diller R, Lamparter T. Unusual Spectral Properties of Bacteriophytochrome Agp2 Result from a Deprotonation of the Chromophore in the Red-absorbing Form Pr. *J Biol Chem.* 2013; 288:31738–31751. [PubMed: 24036118]
41. Ihalaainen JA, Gustavsson E, Schröder L, Donnini S, Lehtivuori H, Isaksson L, Thöing C, Modi V, Berntsson O, Stucki-Buchli B, et al. Chromophore-Protein Interplay During the Phytochrome Photocycle Revealed by Step-Scan FTIR Spectroscopy. *J Am Chem Soc.* 2018; doi: 10.1021/jacs.8b04659 [• Based on vibrational spectroscopy and molecular dynamics simulations, this study provided new insights into the chromophore environment during the transition from Pr to Pfr state. The work suggested that the biliverdin D-ring carbonyl interactions has a major role in destabilizing the β -hairpin conformation of the PHY-tongue element and promoting the transition from Meta-R to Pfr state.]
42. Schmidt A, Sauthof L, et al. Structural snapshot of a bacterial phytochrome in its functional intermediate state. *Nat Commun.* 2018 [•• Exciting new structural insights for the bathyphytochrome Agp2 in its dark-adapted Pfr state as well as intermediates on the way to Pr. Common structural features with other bacteriophytochrome species are observed, but at the same time also the N-terminal region is shown to be affected by biliverdin isomerization and formation of photocycle intermediates]
43. Stojkovi EA, Toh KC, Alexandre MTA, Baclayon M, Moffat K, Kennis JTM. FTIR Spectroscopy Revealing Light-Dependent Refolding of the Conserved Tongue Region of Bacteriophytochrome. *J Phys Chem Lett.* 2014; 5:2512–2515. [PubMed: 25126387]

44. Takala H, Lehtivuori HK, Berntsson O, Hughes A, Nanekar R, Niebling S, Panman M, Henry L, Menzel A, Westenhoff S, et al. On the (un)coupling of the chromophore, tongue interactions, and overall conformation in a bacterial phytochrome. *J Biol Chem*. 2018; 293:8161–8172. [PubMed: 29622676] [• This work reported the three-dimensional structure of the photosensory module of a *DrBphP* variant, which features the chromophore environment of a Pr-state while the PHY-tongue element supported the α -helical conformation observed for Pfr-state structures. The study highlighted the intricate coupling between different functional elements that were shown to be crucial for efficient signal transduction and activation of the output effector.]
45. Buhrke D, Kuhlmann U, Michael N, Hildebrandt P. The Photoconversion of Phytochrome Includes an Unproductive Shunt Reaction Pathway. *ChemPhysChem*. 2018; 19:566–570. [PubMed: 29327408] [•• Using vibrational spectroscopy to characterize the bacteriophytochrome model systems Agp1 and Agp2, the authors revealed the existence of unproductive shunt reactions, from the Meta-R population back to the Pr ground state. Therefore, this study further highlighted an additional phytochrome property that can be used to tune quantum efficiency for light state formation.]
46. Kirpich JS, Mix LT, Martin SS, Rockwell NC, Lagarias JC, Larsen DS. Protonation Heterogeneity Modulates the Ultrafast Photocycle Initiation Dynamics of Phytochrome Cph1. *J Phys Chem Lett*. 2018; 9:3454–3462. [PubMed: 29874080]
47. Björling A, Berntsson O, Lehtivuori H, Takala H, Hughes AJ, Panman M, Hoernke M, Niebling S, Henry L, Henning R, et al. Structural photoactivation of a full-length bacterial phytochrome. *Sci Adv*. 2016; 2:e1600920–e1600920. [PubMed: 27536728] [•• Time-resolved x-ray scattering studies of full-length *DrBphP* identified smaller scale overall structural rearrangements compared to earlier indications based on the isolated photosensory module. This highlighted the important cross-talk of sensor and effector modules and is in line with studies of other full-length bacteriophytochromes.]
48. Li H, Zhang J, Vierstra RD, Li H. Quaternary organization of a phytochrome dimer as revealed by cryoelectron microscopy. *Proc Natl Acad Sci*. 2010; 107:10872–10877. [PubMed: 20534495]
49. Kacprzak S, Njirima I, Renz A, Feng J, Reijerse E, Lubitz W, Krauss N, Scheerer P, Nagano S, Lamparter T, et al. Intersubunit distances in full-length, dimeric, bacterial phytochrome Agp1, as measured by pulsed electron-electron double resonance (PELDOR) between different spin label positions, remain unchanged upon photoconversion. *J Biol Chem*. 2017; 292:7598–7606. [PubMed: 28289094]
50. Loughlin PC, Duxbury Z, Mugerwa TTM, Smith PMC, Willows RD, Chen M. Spectral properties of bacteriophytochrome AM1_5894 in the chlorophyll d-containing *cyanobacterium Acaryochloris marina*. *Sci Rep*. 2016; 6
51. Kim TH, Mehrabi P, Ren Z, Sljoka A, Ing C, Bezginov A, Ye L, Pomès R, Prosser RS, Pai EF. The role of dimer asymmetry and protomer dynamics in enzyme catalysis. *Science*. 2017; 355
52. Neiditch MB, Federle MJ, Pompeani AJ, Kelly RC, Swem DL, Jeffrey PD, Bassler BL, Hughson FM. Ligand-Induced Asymmetry in Histidine Sensor Kinase Complex Regulates Quorum Sensing. *Cell*. 2006; 126:1095–1108. [PubMed: 16990134]
53. Takala H, Niebling S, Berntsson O, Björling A, Lehtivuori H, Häkkinen H, Panman M, Gustavsson E, Hoernke M, Newby G, et al. Light-induced structural changes in a monomeric bacteriophytochrome. *Struct Dyn*. 2016; 3:054701. [PubMed: 27679804]
54. Aldridge ME, Satyshur KA, Anstrom DM, Forest KT. Structure-guided Engineering Enhances a Phytochrome-based Infrared Fluorescent Protein. *J Biol Chem*. 2012; 287:7000–7009. [PubMed: 22210774]
55. Shcherbakova DM, Balaban M, Emelyanov AV, Brenowitz M, Guo P, Verkhusha VV. Bright monomeric near-infrared fluorescent proteins as tags and biosensors for multiscale imaging. *Nat Commun*. 2016; 7 [• Generation of improved versions of bacteriophytochrome-based fluorescent proteins - monomeric near-infrared fluorescent proteins. Their increased brightness and their monomeric state enable comparable applications to GFP-based approaches and allow non-invasive visualization of diverse biological processes.]
56. Yuan C, Li H-Z, Tang K, Gärtner W, Scheer H, Zhou M, Zhao K-H. Near infrared fluorescent biliproteins generated from bacteriophytochrome AphB of *Nostoc* sp. PCC 7120. *Photochem Photobiol Sci*. 2016; 15:546–553. [PubMed: 27004456]

57. Yu D, Baird MA, Allen JR, Howe ES, Klassen MP, Reade A, Makhijani K, Song Y, Liu S, Murthy Z, et al. A naturally monomeric infrared fluorescent protein for protein labeling in vivo. *Nat Methods*. 2015; 12:763–765. [PubMed: 26098020]
58. Yu D, Dong Z, Gustafson WC, Ruiz-González R, Signor L, Marzocca F, Borel F, Klassen MP, Makhijani K, Royant A, et al. Rational design of a monomeric and photostable far-red fluorescent protein for fluorescence imaging *in vivo*: Rational Design of a Monomeric and Photostable Far-Red FP. *Protein Sci*. 2016; 25:308–315. [PubMed: 26549191]
59. Tchekanda E, Sivanesan D, Michnick SW. An infrared reporter to detect spatiotemporal dynamics of protein-protein interactions. *Nat Methods*. 2014; 11:641. [PubMed: 24747815]
60. Pandey N, Nobles CL, Zechiedrich L, Maresso AW, Silberg JJ. Combining Random Gene Fission and Rational Gene Fusion To Discover Near-Infrared Fluorescent Protein Fragments That Report on Protein–Protein Interactions. *ACS Synth Biol*. 2015; 4:615–624. [PubMed: 25265085]
61. Chen M, Li W, Zhang Z, Liu S, Zhang X, Zhang X-E, Cui Z. Novel near-infrared BiFC systems from a bacterial phytochrome for imaging protein interactions and drug evaluation under physiological conditions. *Biomaterials*. 2015; 48:97–107. [PubMed: 25701035]
62. Filonov GS, Verkhusha VV. A Near-Infrared BiFC Reporter for In Vivo Imaging of Protein-Protein Interactions. *Chem Biol*. 2013; 20:1078–1086. [PubMed: 23891149]
63. Piatkevich KD, Subach FV, Verkhusha VV. Far-red light photoactivatable near-infrared fluorescent proteins engineered from a bacterial phytochrome. *Nat Commun*. 2013; 4
64. Rumyantsev KA, Shcherbakova DM, Zakharova NI, Emelyanov AV, Turoverov KK, Verkhusha VV. Minimal domain of bacterial phytochrome required for chromophore binding and fluorescence. *Sci Rep*. 2016; 5
65. Yao J, Kaberniuk AA, Li L, Shcherbakova DM, Zhang R, Wang L, Li G, Verkhusha VV, Wang LV. Multiscale photoacoustic tomography using reversibly switchable bacterial phytochrome as a near-infrared photochromic probe. *Nat Methods*. 2016; 13:67–73. [PubMed: 26550774] [•• Unconventional application of a bacteriophytochrome-based tool combining its reversible photoswitching capacity with photoacoustic tomography. The improved sensitivity and increased penetration depth allowed monitoring tumor growth in living cells with high resolution]
66. Li L, Shemetov AA, Baloban M, Hu P, Zhu L, Shcherbakova DM, Zhang R, Shi J, Yao J, Wang LV, et al. Small near-infrared photochromic protein for photoacoustic multi-contrast imaging and detection of protein interactions in vivo. *Nat Commun*. 2018; 9
67. Wagner JR, Zhang J, von Stetten D, Günther M, Murgida DH, Mroginiski MA, Walker JM, Forest KT, Hildebrandt P, Vierstra RD. Mutational Analysis of *Deinococcus radiodurans* Bacteriophytochrome Reveals Key Amino Acids Necessary for the Photochromicity and Proton Exchange Cycle of Phytochromes. *J Biol Chem*. 2008; 283:12212–12226. [PubMed: 18192276]
68. Ryu M-H, Gomelsky M. Near-infrared Light Responsive Synthetic c-di-GMP Module for Optogenetic Applications. *ACS Synth Biol*. 2014; 3:802–810. [PubMed: 24926804]
69. Hu Y, Wu Y, Mukherjee M, Cao B. A near-infrared light responsive c-di-GMP module-based AND logic gate in *Shewanella oneidensis*. *Chem Commun*. 2017; 53:1646–1648.
70. Reichhart E, Ingles-Prieto A, Tichy A-M, McKenzie C, Janovjak H. A Phytochrome Sensory Domain Permits Receptor Activation by Red Light. *Angew Chem Int Ed*. 2016; 55:6339–6342. [• Using the photosensory module of Cph1, a red light inducible homodimerization system was developed that can be used for the in vivo stimulation of receptor tyrosine kinases in mammalian tissue]
71. Kaberniuk AA, Shemetov AA, Verkhusha VV. A bacterial phytochrome-based optogenetic system controllable with near-infrared light. *Nat Methods*. 2016; 13:591–597. [PubMed: 27159085]
72. Redchuk TA, Omelina ES, Chernov KG, Verkhusha VV. Near-infrared optogenetic pair for protein regulation and spectral multiplexing. *Nat Chem Biol*. 2017; 13:633. [PubMed: 28346403] [• Elegant approach to optimize a red light inducible heterodimerization system based on the natural interaction of a bacteriophytochrome with its cognate transcriptional regulator.]
73. Redchuk TA, Karasev MM, Omelina ES, Verkhusha VV. Near-Infrared Light-Controlled Gene Expression and Protein Targeting in Neurons and Non-neuronal Cells. *ChemBioChem*. 2018; 19:1334–1340. [PubMed: 29465801]

74. Cai Y, Su M, Ahmad A, Hu X, Sang J, Kong L, Chen X, Wang C, Shuai J, Han A. Conformational dynamics of the essential sensor histidine kinase WalK. *Acta Crystallogr Sect Struct Biol*. 2017; 73:793–803.

Figure 1. The remarkable modularity of phytochrome sensors.

(a) Bacteriophytochromes typically feature a PAS-GAF-PHY domain arrangement (top right), containing an additional N-terminal segment (NTS). Exceptions are cyanobacterial Cph1-like bacteriophytochrome members that use a cysteine residue of the GAF domain to covalently link their cognate phycocyanobilin cofactor. The photosensory module (PSM) can be linked to a series of different effector domain arrangements that are involved in bacterial second messenger metabolism (GGDEF and EAL domains) or phosphorylation (HKs and the HWE-subfamily of HKs together with their cognate response regulators (RR)) and dephosphorylation (PPM – metal dependent Ser/Thr protein phosphatases) reactions. In addition, PAS-domain containing bacteriophytochromes and potentially also the subfamily without enzymatic effector are involved in protein-protein interactions or protein-ligand

interactions. For simplicity, domain architectures are shown for single phytochrome chains, however, dimerization of individual protomers plays an important role in functional signaling of all phytochrome families (*cf.* Figure 2).

For comparison, the unrooted tree illustrating individual bacteriophytochrome subfamilies also contains distantly related phytochrome families characterized by the presence of GAF and PHY domains (GAF-only cyanobacteriochromes were omitted for simplification). Fungal phytochromes (bottom left) are characterized by almost identical domain arrangements of their PSM modules, however, feature more extensive N-terminal extensions (NTE). Another family of bacterial origin are the Cph2-like phytochromes (middle left) that have lost their PAS domain and use a different cysteine residue of the GAF module to covalently link to their cognate bilin cofactor. Last but not least, plant phytochromes (top left) also use the same cysteine residue to interact with their bilins as the latter family, while still containing a PAS domain and an NTE. Representative domain architectures of frequently observed effector modules are shown for each phytochrome family. In the case of Cph2, the additional bilin-containing GAF domain and the second GGDEF module are not shown explicitly. Uniprot accession numbers of individual sequences used for the unrooted tree together with technical details of tree generation are provided as Supplementary Data.

(b) Bacteriophytochromes exhibit different photoequilibria upon red light illumination, as observed from the superposition of absorption spectra from two representative members. *DrBphP* and *IsPadC* absorption spectra in their dark-adapted Pr forms and red-light activated states are scaled based on the 280 nm absorption corrected for the extinction coefficients of the holoproteins. *DrBphP* is represented as solid lines while *IsPadC* is represented in dashed lines. While the first coordination shell of the BV environment is highly conserved, differences in the NTS and PHY-tongue regions enable substantially different properties in extinction coefficients or mixed Pfr/Meta-R states in different functional states. **(c)** Chemical structures of *5Z_{syn}*, *10Z_{syn}*, *15Z_{anti}* biliverdin and its *5Z_{syn}*, *10Z_{syn}*, *15E_{anti}* conformation including the characteristic Cys-linkage. Depending on the protein environment either the *15Z* form is stabilized in the dark-adapted form (Pr – canonical bacteriophytochromes) or the *15E* configuration corresponds to the dark state in bathyphytochromes (Pfr). Interconversion between the two forms can either be triggered by red or far-red light, respectively, or by thermal processes from the light activated state back to the dark-adapted state.

Figure 2. Critical structural features of bacteriophytochrome function.

(a) For the purpose of showing Pr and Pfr states in the context of a full-length system, we chose the asymmetric Pr/Pfr heterodimer of *IsPadC* (pdb 6et7, [24]) as model system. The Pr protomer is colored according to Figure 1a with different hues of red for the PAS, GAF and PHY domains, grey for the coiled-coil sensor-effector linker and orange for the GGDEF output domain. The Pfr protomer is shown in lighter colors including a transparent surface representation. The BV molecules are represented as blue and purple stick models for the Pr and Pfr protomers, respectively. (b) Close up views of the N-terminal segment (NTS) and its covalent attachment to biliverdin via the conserved Cys-residue and the C3² atom, respectively, for three systems structurally corresponding to Pr and four systems for Pfr. All structures are superposed based on their PAS-GAF domains and C α -backbone atoms of one protomer. The systems are *IsPadC* (pdb 5llw, orange), *AfAgp1* (pdb 5hsq, blue), *SaBphP* (pdb 6bao, light blue) and *IsPadC* (Pfr protomer of pdb 6et7, orange), *RpBphP1* (pdb 4gw9, cyan), *PaBphP* (pdb 3nhq, off-white), *AfAgp2* (pdb 6glz, green), respectively. Interesting differences in the conformation of the NTS can be observed depending on the activation state of the biliverdin. The Cys-residue approaches the biliverdin from its β -side in Pr (left part), whereas it links to BV from the α -side in Pfr (right part). These different conformations are accompanied by reorganization of the NTS and the PHY-tongue. Interestingly, *DrBphP* appears to have a rather rigid NTS that does not show conformational changes induced by light activation, however, subtle changes in the mode of cofactor attachment correlating with other systems can be observed (cf. Figure 3b). (c) Different view

of the same composition as in panel b, highlighting the differences in conformation of the NTS and the PHY-tongue between Pr (left) and Pfr (right) states. **(d)** Direct comparison of the BV environments of Pr and Pfr states for the *IsPadC* system. Highly conserved bacteriophytochromes residues that are structurally affected by light activation are shown as stick models and indicated specifically. For better orientation, darker colors correspond to the NTS and lighter colors to the PHY tongue element; orange is used for Pr of *IsPadC* and red for its Pfr state. The BV cofactors for Pr and Pfr states are shown as blue and purple stick models, respectively. **(e)** The characteristic figure-of-eight knot of phytochromes. Hydrophobic residues line the site where the NTS is threaded through the knot-lasso region of the GAF domain. Seven different systems are superposed based on their PAS-GAF domains and highlight the rigid positioning of the NTS segment via strictly conserved contacts between Gln29 (*IsPadC* numbering) and the backbone of the GAF domain, as well as Arg246 of the knot-lasso with the propionate moiety of the BV B-ring. Substantially different conformations of Pr and Pfr states N-terminal to the conserved Ile22 residue do not affect the conformation of the knot region. Pr and Pfr associated BVs are colored as in panel d. The bacteriophytochrome structures used in this comparison correspond to *IsPadC* (pdb 5llw, orange), *SaBphP* (6bao, light-blue), *AfAgp1* (pdb 5hsq, blue), *DrBphP* (pdb 5mg1, dark blue) for Pr and *PaBphP* (pdb 3nhq, off-white), *RpBphP1* (pdb 4gw9, cyan) and *AfAgp2* (pdb 6glz, green) for Pfr. **(f)** The central helical spine connects the PAS-GAF bidomain and the PHY domain and continues to downstream effector domains via coiled-coil sensor-effector linkers. Three structures of bacteriophytochromes are shown to highlight the differences in parallel dimer interfaces also in the context of full-length Pr systems. Cartoon models of *IsPadC* (pdb 5llw, orange), *XccBphP* (pdb 5akp, palecyan) and the *DrBphP*-based artificial PaaC system (pdb 6fht, brown) are shown with one protomer of each assembly represented in lighter colors. While individual PAS-GAF bidomains superpose very well, the whole PSM in its dimeric state was used for superposition in this case and highlights the substantially different architectures at the central helical spine, even in the presence of downstream effectors. Different relative orientations of the PHY dimers and altered interactions of the PHY-tongues with the GAF domains consequently enable different structural rearrangements of the output domains. More detailed comparisons of similar interpretations are described in refs. [7,18].

Figure 3. Overview of signal transduction mechanisms in full-length bacteriophytochromes.

(a) Schematic representation of overall signaling mechanisms in full-length bacteriophytochromes, for which structural information on Pr and Pfr states of the phytochrome sensor are available. *LsPadC* (pdb 5llw [7], 6et7 [24]), *DrBphP-HK* (pdb 4o01 [15]), and *DrBphP-Cya2* (pdb 6fht [16]) systems are represented as surface models with the central helical spine, PHY-tongue, and sensor-effector linker regions highlighted in cartoon representation. For *LsPadC* and *DrBphP*, the activated state structure of the phytochrome dimer is superposed to the Pr-form based on the PAS-GAF core. Domains of the phytochrome sensor are colored according to Figure 1a. Output effector domains are colored according to their subfamily type in Figure 1a. The artificial effector of the fusion between *DrBphP* and *Synechocystis* Cya2 is colored in brown [16]. 15Z and 15EBV are represented in sticks and colored in blue and purple, respectively. All three systems feature a similar

PHY-tongue refolding event upon BV isomerization. However, the proposed signal transduction processes affect the phytochrome dimer interface differently and ultimately regulate the activity of the associated output effectors based on diverse molecular mechanisms. A simplified translation of the coiled-coil linker is observed for *IsPadC* upon asymmetric activation of its phytochrome sensor, whereas a symmetric activation of the *DtBphP* phytochrome leads to a rotational motion of the HK domains in *DtBphP*-HK, or alternatively an increase in conformational dynamics due to the destabilization of secondary structure in the sensor-effector linker region in *DtBphP*-Cya2. **(b)** Close-up views of PHY-tongue refolding upon 15*E* isomerization of BV together with top-views of the respective effector domains. The superposition of different GGDEF output conformations (pdb 5llw [7], grey and pdb 6et7 [24], red) of *IsPadC* showed an increase in asymmetry of the GGDEF dimer. The superposition of inactive ([47], grey) and active conformations (pdb 4u7o [74], red) of the HK, based on the models of full length *DtBphP* [47], shows the rotational motion of the HK domains suggested by modelling of full-length *DtBphP* [47]. With respect to AC/GC activation, light activation modulates the dynamics of the interaction of the linker helices with the cyclase transducer elements and characteristic elements of the cyclase core, eventually releasing the output from an inhibited conformation [16].

Figure 4. Fluorescent proteins and optogenetic systems based on bacteriophytochromes. (a) NIR FPs are available as dimers or monomers and mostly feature engineered CBDs. Rational design of blue-shifted FPs is facilitated by introduction of Cys in a conserved SPXH motif of the GAF domain. A small single domain FP was developed with GAF-FP. Other than most FPs, photoactivatable FPs consist of the whole PSM to enable photoactivation by Pfr to Pr transformation. (b) Several split probes for fluorescence complementation have been developed based on IFP and iRFPs. The split sites are usually situated in the extended loop between PAS and GAF domains or in the C-terminal region of

the PAS domain. Similar strategies were also employed in the generation of *DtSplit*, which is not based on fluorescent properties of *DtBphP*, but its characteristic absorption. **(c)** Engineered photoactivatable enzymatic functionalities include cyclases (AC/GC), phosphodiesterases (PDE) and diguanylyl cyclases (GGDEF). **(d)** The far-red light-dependent heterodimerization of *RpBphP1* and *RpPpsR2* was exploited in several protein targeting experiments and optimized systems have been developed by truncation of *RpPpsR2* to the essential Q-PAS1 fragment and its combination with *AsLOV2*. For specific applications, either interaction partner can be tagged with localization sequences and/or target proteins of interest.