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PATAN-domain regulators interact with the Type IV pilus motor to control phototactic orientation in the cyanobacterium *Synechocystis*

Yu Han | Annik Jakob | Sophia Engel | Annegret Wilde  | Nils Schuergers

Molecular Genetics, Institute of Biology III, University of Freiburg, Freiburg, Germany

Correspondence

Annegret Wilde, Molecular Genetics, Institute of Biology III, University of Freiburg, 79104 Freiburg, Germany.
Email: annegret.wilde@biologie.uni-freiburg.de

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Abstract

Many prokaryotes show complex behaviors that require the intricate spatial and temporal organization of cellular protein machineries, leading to asymmetrical protein distribution and cell polarity. One such behavior is cyanobacterial phototaxis which relies on the dynamic localization of the Type IV pilus motor proteins in response to light. In the cyanobacterium *Synechocystis*, various signaling systems encompassing chemotaxis-related CheY- and PatA-like response regulators are critical players in switching between positive and negative phototaxis depending on the light intensity and wavelength. In this study, we show that PatA-type regulators evolved from chemosensory systems. Using fluorescence microscopy and yeast two-hybrid analysis, we demonstrate that they localize to the inner membrane, where they interact with the N-terminal cytoplasmic domain of PilC and the pilus assembly ATPase PilB1. By separately expressing the subdomains of the response regulator PixE, we confirm that only the N-terminal PATAN domain interacts with PilB1, localizes to the membrane, and is sufficient to reverse phototactic orientation. These experiments established that the PATAN domain is the principal output domain of PatA-type regulators which we presume to modulate pilus extension by binding to the pilus motor components.

KEYWORDS

chemotaxis, CheY, PATAN, phototaxis, type IV pili

1 | INTRODUCTION

Phototaxis allows cyanobacteria to seek out optimal conditions for their photosynthetic lifestyle. Depending on the quality and intensity of illumination, single-cell and whole-cell colonies alike can move either toward (positive phototaxis) or away (negative phototaxis) from a light source (Choi et al., 1999; Ng et al., 2003). This behavior requires the intricate spatial and temporal coordination of the motility machinery. The single-celled cyanobacterium *Synechocystis*

sp. PCC 6803 (hereafter *Synechocystis*) is a model for studying the mechanisms of phototaxis control in cyanobacteria. During phototaxis, *Synechocystis* establishes directionality by focusing the incoming illumination within its lens-like cell body. The local excitation of receptors in the focal spot is thought to guide the asymmetrical distribution of PilB1, resulting in polar pilus activity and phototactic movement in regard to the light vector (Nakane & Nishizaka, 2017; Schuergers et al., 2015, 2016). The integration of different light signals governs phototactic orientation. Wild-type cells exhibit

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positive phototaxis toward wavelengths ranging from green to far-red, whereas blue or UV light inhibits motility or induces negative phototaxis (Choi et al., 1999; Ng et al., 2003; Savakis et al., 2012).

Like many bacteria, *Synechocystis* moves along solid surfaces using type IVa pili (TFP), protein filaments that also play a role in adhesion, aggregation, and DNA uptake (Bhaya et al., 2000; Conradi et al., 2019; Schuergers & Wilde, 2015; Yoshihara et al., 2001). Studies on other bacteria demonstrated that TFP-mediated "twitching" motility relies on the sequential extension, surface adhesion, and retraction of the pilus filament to pull the cell forward (Merz et al., 2000). Translocation of the pilus through a complex that spans the cell envelope is driven by the inner membrane protein PilC that promotes the (dis-)assembly of the pilus that is composed of major and minor pilin subunits (Takhar et al., 2013). Two antagonistically working motor ATPases can interact with PilC at the cytoplasmic face of the inner membrane to power cell movement (Chang et al., 2016; McCallum et al., 2017). In an ATP-driven process, PilB facilitates pilus assembly while PilT is required for pilus retraction (Craig et al., 2019). In *Synechocystis*, which encodes two PilB and PilT homologs, PilB1 and PilT1 fulfill these functions (Bhaya et al., 2000; Okamoto & Ohmori, 2002; Yoshihara et al., 2001). Although a second retraction ATPase PilT2 was reported to be crucial for positive phototaxis (Bhaya et al., 2000), its exact function has not been elucidated. A gene encoding an additional PilB homolog (PilB2) is dispensable for phototaxis (Yoshihara et al., 2001).

Different signal transduction pathways regulate pilus assembly or control the reversal of cell polarity (i.e., the switch from positive to negative phototaxis). The most prevalent systems in cyanobacteria are homologous to the chemosensory systems that regulate flagellar rotation in many chemotactic bacteria. The sensor of such a canonical system is a methyl-accepting chemotaxis protein (MCP) that transduces a chemical cue to the histidine kinase CheA, which is linked to the MCP via CheW. Upon autophosphorylation of CheA, the phosphoryl group is transferred to the response regulator CheY that diffuses to the base of the flagellum and promotes a switch in the direction of flagellar rotation (Porter et al., 2011). Homologous systems in cyanobacteria typically harbor two CheY-like response regulators but miss the CheR and CheB proteins that modulate MCP methylation and temporal adaptation in canonical systems (Wuichet & Zhulin, 2010). *Synechocystis* encodes three of these systems, designated Tax1-3, for their role in phototaxis (Bhaya et al., 2001a) (Figure 1a,b). The Tax1 system with the blue-/green-absorbing cyanobacteriochrome photoreceptor PixJ1 is essential for positive phototaxis, and disruption of *pixJ1* and the response regulator genes *pixG* or *pixH* leads to negative phototaxis in response to white light (Bhaya et al., 2001a; Ng et al., 2003; Yoshihara et al., 2000, 2004). The MCPs of the Tax2 and Tax3 systems lack a discernible photoreceptor domain, and it is unclear which signals they can perceive. However, recent findings show that the Hmp chemotaxis system of *Nostoc punctiforme* (*N. punctiforme*) (homologous to *Synechocystis* Tax3) might be capable of sensing light indirectly, possibly via alterations in proton

motive force (Harwood et al., 2021). Although disruption of the Tax2 system does not affect motility (Bhaya et al., 2001a), insertions in many components of the Tax3 pathway lead to defects in pilus assembly that abrogate cell movement completely (Bhaya et al., 2001a; Chung et al., 2001; Yoshihara et al., 2002). Notably, the Tax3 system is linked to pilus assembly and/or retraction; insertional mutations in sequences encoding the second response regulator (*pilH*) or the phosphotransfer domain of the CheA homolog (*slr0073*) encoded by the *tax3* gene cluster cause a hyper-piliated phenotype, whereas insertions near the dimerization domain of CheA (*slr0332*), or within the MCP-homolog (*slr1044*) lead to a loss of TFP on the cell surface (Yoshihara et al., 2002). Thus far, it is unresolved how the Tax1 and Tax3 signaling pathways transduce their respective signals to the motility machinery.

Homologous chemotaxis-like systems control phototaxis in other cyanobacterial species as well. Motility in hormogonia of *N. punctiforme* depends on the Hmp system, while the Ptx system (homologous to the *Synechocystis* Tax1 system) is essential for directed phototaxis (Campbell et al., 2015; Risser et al., 2014). In a recently isolated motile *Synechococcus elongatus* strain UTEX 3055, the so-called Pix system was also shown to regulate both positive and negative phototaxis (Yang et al., 2018).

Two additional systems which involve CheY-like response regulators but lack the other typical chemotaxis regulatory components, control phototactic orientation in *Synechocystis* (Figure 1a,b). The two-component regulatory system UirS–UirR triggers negative phototaxis in response to UV-A light by inducing the expression of the response regulator LsiR (Song et al., 2011). The cyanobacteriochrome UirS is also able to sense ethylene, which represses the transcription of *lsiR*, resulting in a more robust positive phototaxis response (Kuchmina et al., 2017; Lacey & Binder, 2016). The photoreceptor PixD interacts with the response regulator PixE to form complexes that dissociate under high-intensity blue-light irradiation (Masuda et al., 2004; Yuan & Bauer, 2008). Unbound PixE is thought to reverse phototactic orientation and is crucial for the polar assembly of TFP during negative phototaxis under intense blue illumination (Nakane & Nishizaka, 2017; Okajima et al., 2005). While the molecular function of LsiR is enigmatic, it was recently established that PixE interacts with the motor ATPase PilB1 (Jakob et al., 2019).

An intriguing feature of these signaling pathways is the specific domain structure of the CheY-like response regulators. The typical cyanobacterial chemotaxis systems each contain two response regulators. While one comprises only the archetypical CheY receiver domain (REC), the other has an N-terminal PATAN domain (which corresponds to DUF4388/PF14332 in the PFAM database [Mistry et al., 2021]) fused to the REC domain (Figure 1b). In *Synechocystis*, this bipartite domain structure (PATAN-REC) is also found in the response regulators PixE, LsiR, and in a so far uncharacterized CheY-like protein Slr1594 (Bhaya et al., 2001a). This architecture is the hallmark of PatA-type response regulators (Makarova et al., 2006) named after the *Anabaena* sp. PCC 7120 (*Anabaena*) PatA protein that is required for the accurate patterning of nitrogen-fixing heterocysts along cell filaments (Liang

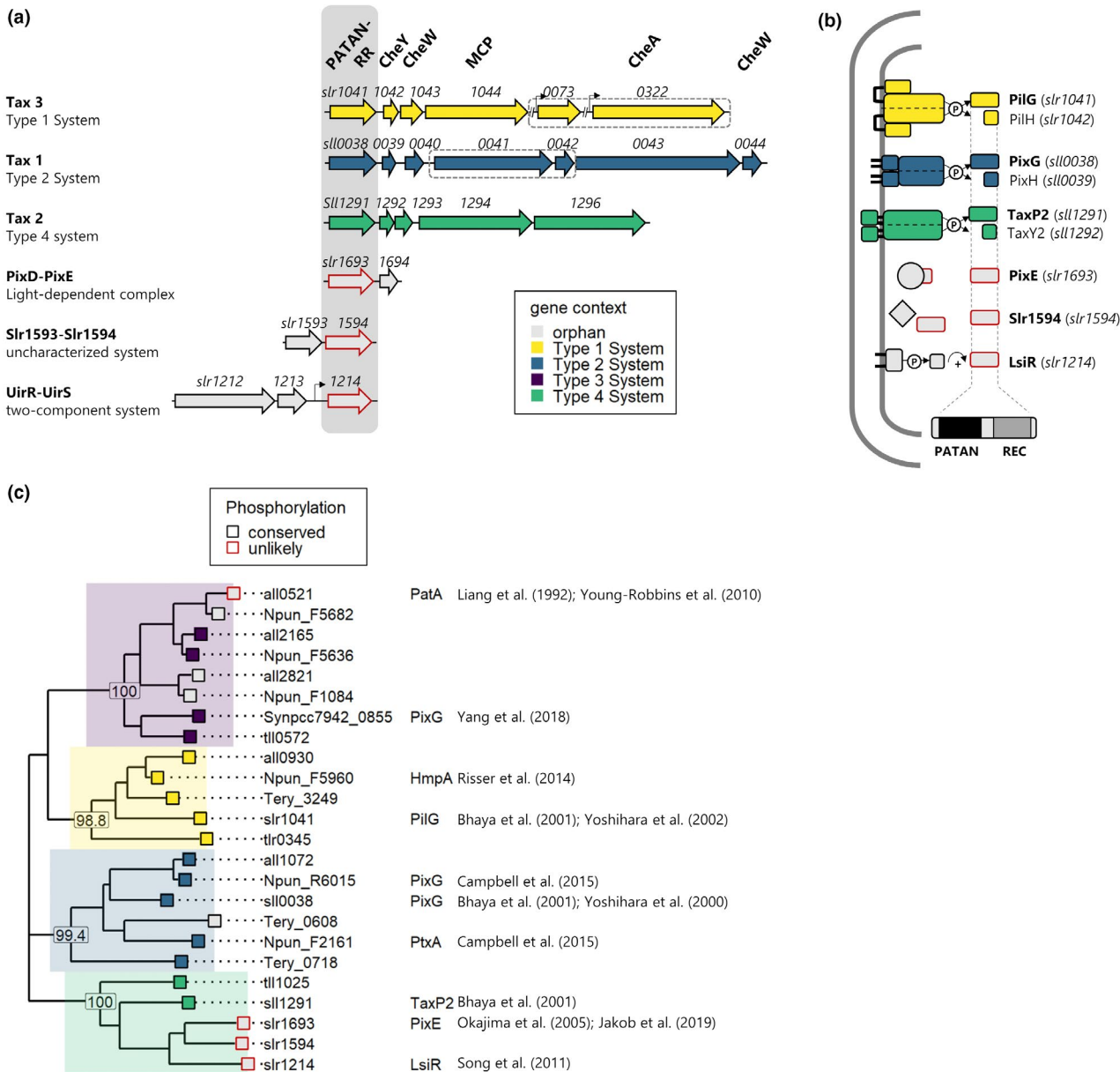


FIGURE 1 Cyanobacterial PatA-type response regulators originate from ancestral chemosensory systems. (a) The genome of *Synechocystis* encodes six PatA-type response regulators comprising an N-terminal PATAN domain and a C-terminal REC domain. Three of these are encoded as the first gene in the chemotaxis-like signaling systems Tax1, Tax2, and Tax3. The other three occur in the context of entirely unrelated signaling systems. Arrows indicate additional transcription start sites and dashed lines encircle split genes. (b) Simplified scheme depicting the localization and regulation of PatA-type regulators. The CheA-CheW-MCP components are anchored through transmembrane helices in the MCP proteins (thick lines), which show a variable domain architecture. Type 3 chemotaxis systems are not present in *Synechocystis* but in other cyanobacteria (see panel c). (c) Maximum likelihood phylogenetic tree of PatA-type regulators identified from a sample set of cyanobacterial sequences aligned with MAFFT via Guidance2 with bootstrap probabilities calculated using 500 replicates shown for selected nodes. For PatA-homologs that are part of a chemosensory cluster, the type of the system as determined from the phylogeny of the associated CheA histidine kinases (see Figure S1) is color-coded and corresponds to the respective color in (a) and (b) panels. The outline of the tip symbols indicates if conserved residues required for phosphorylation of the REC domain are conserved (black square). A red square indicates the absence of a conserved phosphorylation site in the REC domain of the protein. For characterized genes or chemosensory systems, the relevant references are given in (c)

et al., 1992; Young-Robbins et al., 2010). PatA localizes to sites of cell division and was recently shown to interact with components of the divisome in *Anabaena* (Valladares et al., 2020; Young-Robbins et al., 2010).

A detailed characterization of a PATAN domain has not been published and its molecular function is still unknown. To gain insight into the role of this domain for the function of PatA-type response regulators, we compared the localization and protein interaction of

the different PatA-type regulators with their REC-only counterparts (CheY-type from here on) and tested the behavior of mutants expressing isolated PATAN domains.

2 | RESULTS

2.1 | Phylogeny of PatA-type response regulators

PatA-type response regulators such as PixE, PixG, or PatA act as output proteins for unrelated signaling pathways, such as phototaxis and heterocyst formation. We inferred a maximum-likelihood tree built from PATAN domain-containing response regulators in a sample set of cyanobacteria to unravel their phylogeny (Figure 1). Considering that most detected homologs represent the first gene within a chemotaxis locus, we additionally constructed a phylogenetic tree for these systems based on the CheA sequences (Figure S1). Wuichet and Zhulin (2003) identified four paralogous chemotaxis clusters (Types 1–4 systems) that harbor MCPs with greatly divergent sensory modules within five cyanobacterial genomes. All PatA-type regulators cluster with high confidence into distinct clades that perfectly match the phylogeny of the four evolutionarily conserved chemotaxis systems (Figure 1c). These systems were classified as chemotaxis systems involved in TFP-based motility (Wuichet & Zhulin, 2010) and except for Type 4 system, there is multiple evidence that these chemotaxis systems indeed regulate twitching motility in cyanobacteria (see References in Figure 1). Importantly, PatA-type regulators that are not part of a chemosensory system—like the eponymous PatA—do not form a monophyletic cluster but are branching from within different clades. An evaluation of the receiver domains suggests that the conserved aspartate or in case of LsiR other residues that are crucial for phosphorylation have been lost in some of these genes (Figure S2). The phylogenetic analysis thus implies that distinct “orphan” PatA-type regulators evolved independently through gene duplication of chemosensory PatA-type proteins in different cyanobacterial lineages. At least some of them were re-wired to new, phosphorylation-independent sensory pathways as is the case in *Synechocystis* (Figure 1b). Here the successive duplications of a Tax2-associated gene lead to the emergence of LsiR, PixE, and a third, so far uncharacterized PatA-type regulator encoded by *slr1594*. Given that all other PATAN domain-containing proteins in *Synechocystis* are part of a chemosensory system or are crucial for reversal of phototactic orientation, it is tempting to speculate that *Slr1594* might have a similar function. As the gene is cotranscribed with an EAL-domain protein (Kopf et al., 2014) which could function in the degradation of c-di-GMP, *Slr1594* might link phototactic orientation to changes in the concentration of this second messenger.

2.2 | PatA-type regulators localize to the cytoplasmic membrane

Previous work determined that PixE partially localizes to the cell membrane and interacts with the motor ATPase PilB1 (Jakob

et al., 2019). To systematically study the subcellular localization of the different PatA-type regulators in *Synechocystis*, we expressed these as fusion proteins containing a C-terminal enhanced yellow fluorescent protein (eYFP). For comparison, we constructed fluorescent versions of the CheY-like PilH and PixH regulators, encoded in the same *tax* gene cluster but which are missing the PATAN domain (Figure 1). To avoid any polar effects on the expression of the chemotaxis operons, we inserted the expression cassette at a neutral site in the chromosome of the wild-type strain under control of the copper-repressed promoter *PpetJ* (Kuchmina et al., 2012). We dropped *Slr1594* from the analysis as the transformation of *Synechocystis* with the expression construct was not successful for unknown reasons. After verifying that fluorescence is specifically induced in a copper-depleted medium (Figure S3), we analyzed the distribution of the fluorescence signal in cells grown on a solid medium using epifluorescence microscopy. Figure 2 clearly shows that all PatA-type fusion proteins localize to the cell periphery. While the orphan PATAN-CheY regulators show a patchy distribution, the ones encoded from a chemotaxis locus are evenly distributed along the cytoplasmic membrane. This is in stark contrast to the PilH-eYFP and PixH-eYFP CheY-type proteins that accumulate mainly in the cytoplasm, with only a very small fraction being visible at the membrane.

2.3 | PatA-type regulators interact with the pilus extension motor

Notwithstanding their divergent evolutionary history, regulators such as PixG, LsiR, or PixE have a remarkably similar function in regulating phototactic orientation, and we hypothesize that the entire PatA protein family might share a conserved mode of action. Considering the membrane localization and the previously established *in vivo* interaction of PixE with the extension motor PilB1 (Jakob et al., 2019), it seems likely that these proteins directly target components of the motility apparatus. We screened possible interactions of the response regulators PilG/H, PixG/H, PixE, and LsiR that are controlling motility with the essential TFP motor components PilB1, PilT1, and the platform subunit PilC using yeast two-hybrid (Y2H) assays (Figure 3 and Figure S4). To include the integral membrane protein PilC in the screen, we generated two constructs to separately express both the N-terminal and C-terminal cytoplasmic domains (PilC_N residues 1–173 and PilC_C 239–377, respectively). Additionally, we included PilT2 in the assay, as this motor ATPase has been shown to interact with PilG in a bacterial two-hybrid assay (Kera et al., 2020).

Collectively, the Y2H assays established that not only PixE but also all tested PatA-type regulators interact with the extension motor ATPase PilB1 but not with the retraction motor ATPase PilT1 nor with PilT2 (Figure 3). We attribute the discrepancy in PilT2–PilG interaction (Kera et al., 2020) with the relatively small overlap and high false-positive rate observed between different two-hybrid methods. Moreover, we detected interactions between PixE, LsiR,

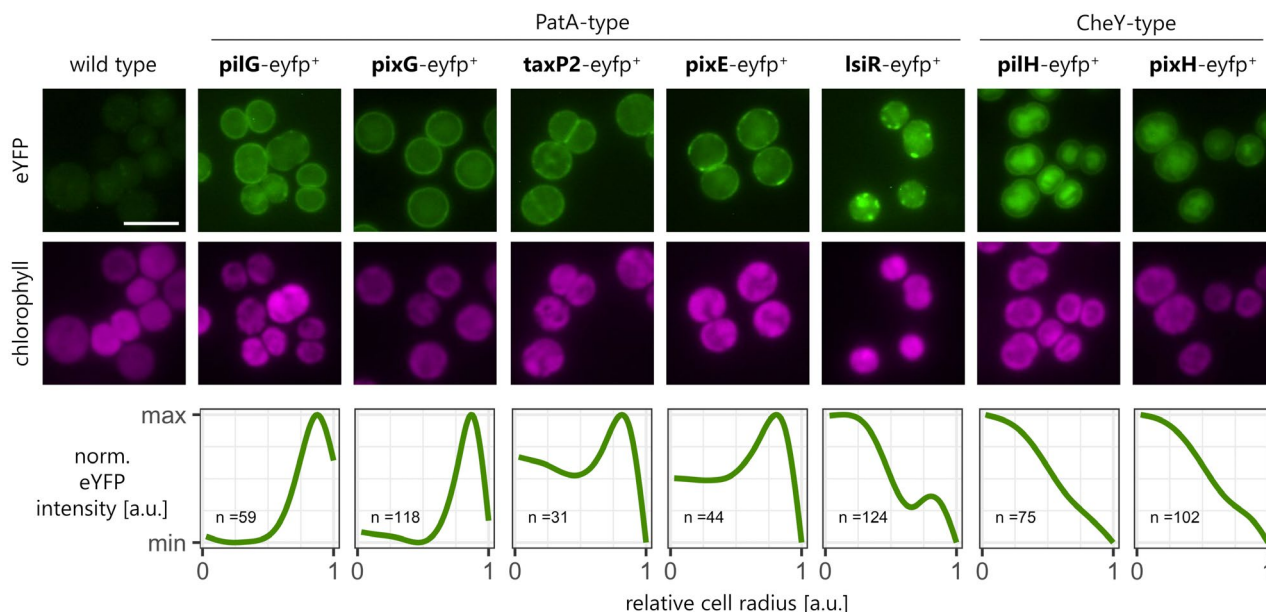


FIGURE 2 PatA-type regulators localize to the inner membrane. (Upper panel) Fluorescence microscopy images show the subcellular localization of PatA-type and CheY-type response regulators involved in the regulation of phototaxis, TFP biogenesis, and adhesion. The proteins harboring a C-terminal eYFP fusion were expressed from a neutral genomic locus in a wild-type *Synechocystis* strain. Cells were grown on 0.5% (w/v) BG11 agar plates without copper to induce gene expression. eYFP fluorescence is shown in green and chlorophyll fluorescence in magenta. Scale bars = 5 μ m. (Lower panel) Relative eYFP fluorescence distribution from cell center to cell periphery. The curves were fitted from the radial intensity profiles of n cells and normalized to the wild-type fluorescence distribution

and PixG and the N-terminal cytoplasmic domain of the platform protein (PilC_N). Except for PixG, all PatA-type regulators showed self-interaction, indicative of a possible multimerization. In contrast to the PATAN domain-containing response regulators, the CheY-type proteins PixH and PilH did not interact with any of the pilus proteins and showed no self-interaction in our analysis. Overall, protein interactions of the orphan regulators LsiR and PixE could be detected under more stringent conditions (i.e., higher 3-AT concentrations) (Figure 3 and Figure S4). It is conceivable that the protein-binding affinity depends on the signaling state of the REC domain, which is most probably phosphorylation independent in the case of the orphan regulators LsiR and PixE. On the other hand, the Pix and Pil response regulators, which are part of a chemotaxis system, cannot be phosphorylated by their cognate histidine kinases because they are not coexpressed in the yeast cells. Therefore, the response regulators might adopt a different signaling state with lower binding affinity in the heterologous host.

Apart from the C-terminal domain of PilC, all tested pilus proteins showed self-interaction. Such a multimerization is in agreement with similar results reported previously and may indicate that the proteins are expressed and correctly folded in yeast. The cytosolic N-terminal domain of *Thermus thermophilus* PilC is known to form a dimer (Karupiah et al., 2010), and self-interaction of the putatively hexameric motor ATPases PilB1 and PilT1 has been verified in *Synechocystis* (Jakob et al., 2019; Schuergers et al., 2014). It is noteworthy that no interaction could be observed between the paralogous PilT proteins, indicating that PilT2, which is conserved among many cyanobacteria (Schuergers & Wilde, 2015) forms

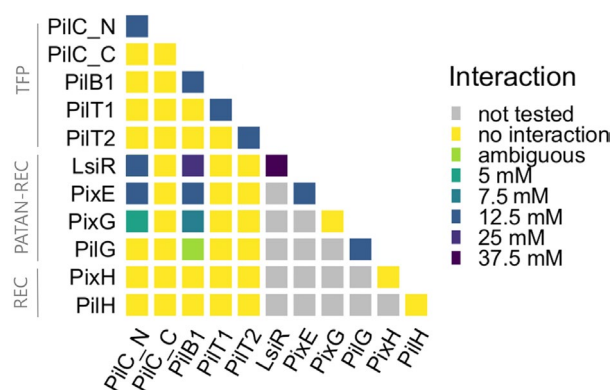


FIGURE 3 PatA-type regulators interact with the TFP extension motor. Interaction matrix of response regulators and TFP components from the inner membrane. Protein-protein interactions were tested using a Y2H screen by transforming yeast strain AH109 with prey (GAL4 AD) and bait (GAL4 BD) vectors and selection on leucine-, tryptophan-, and histidine-depleted selection medium supplemented with different amounts of the competitive inhibitor 3-AT. *Synechocystis* proteins fused C-terminally and N-terminally to BD and AD domains (eight possible combinations for the interaction between two proteins and four combinations for self-interactions) were tested and the highest permissive 3-AT concentration is shown. The PilG-PilB1 interaction is denoted as ambiguous as only four out of six tested yeast clones showed interaction. For representative original Y2H data see Figure S4

separate homomers. Together, these results suggest that the PATAN domain-containing response regulators might alter TFP extension by binding to PilB1 and the pilus platform PilC.

2.4 | The PATAN domain conveys signal output to the TFP

To pinpoint the interacting protein domains, we analyzed the interaction of truncated PixE variants with the PilB1 protein. Similar to full-length PixE, the shortened version containing only the PATAN domain (PixE_{PATAN} residues 1–258) retains its ability to interact with either PilB1 or PilC_{N-term} in the Y2H assay. A protein consisting of only the C-terminal PixE receiver domain (PixE_{REC} residues 251–380) did not show an interaction (Figure 4a). However, we can not exclude that this was due to insufficient expression or lack of post-translational modifications in yeast. Next, we probed the interaction of full-length PixE, as well as the truncated PixE_{PATAN} variant with

different PilB1 constructs to narrow down the interaction surface for the PATAN domain (Figure 4b). TFP extension ATPases are multi-domain proteins encompassing an amino-terminal domain (NTD) and carboxy-terminal domain (CTD) which can be further divided into subdomains N1 to C2 (Yamagata & Tainer, 2007). The interaction surface seems to lie within the N-terminal domain as only a truncated PilB1_{NTD} (residues 1–366) but not the PilB1_{CTD} (residues 367–672) variant showed interaction in Y2H assays.

Considering the interactions with PilB1 and PilC_(N-term), we assume that the PATAN domain localizes the response regulator to the pilus base at the inner membrane. We evaluated the subcellular localization of the PixE PATAN domain which we fused to eYFP using the same strategy as for the full-length PixE. The micrographs in

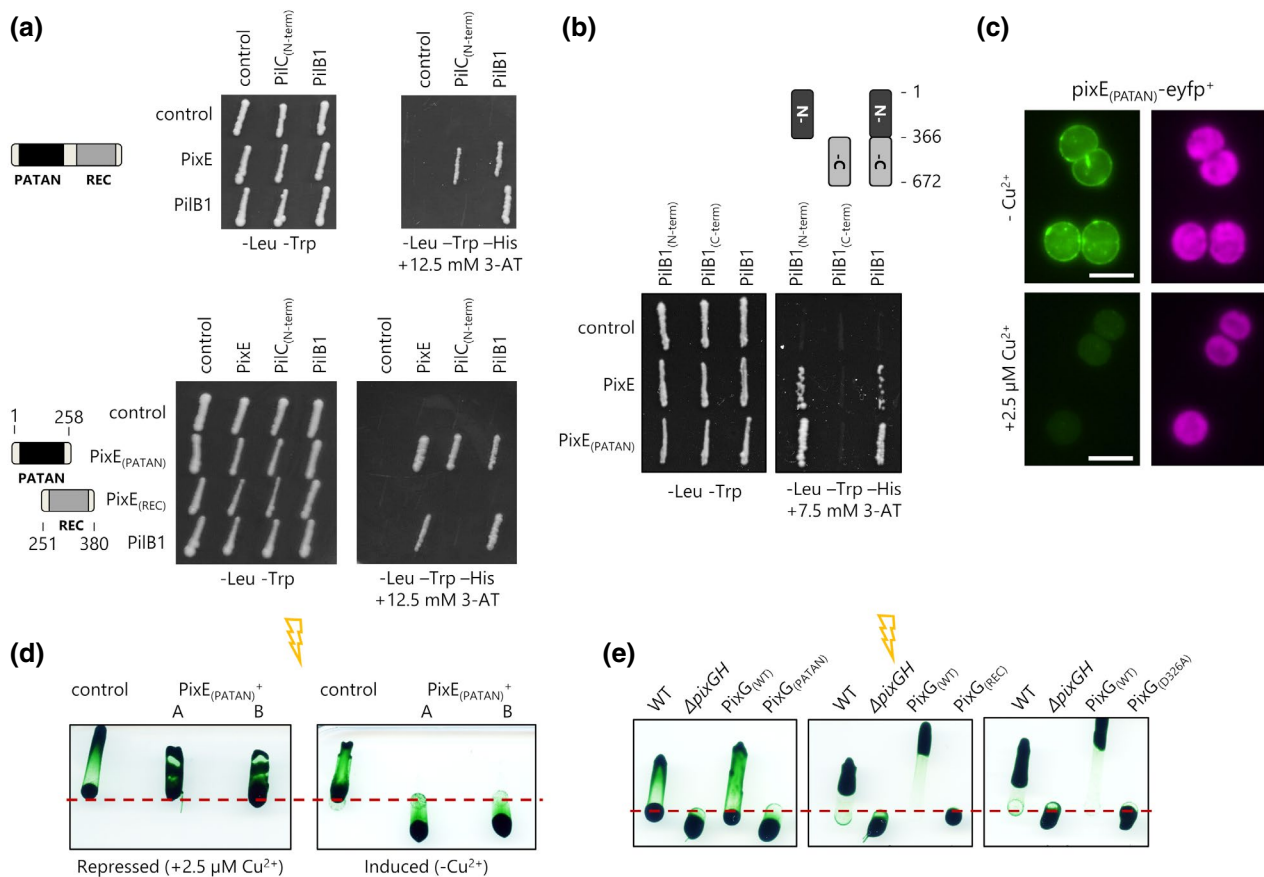


FIGURE 4 The PATAN domain alone is sufficient to mediate interaction with PilB1 and to switch phototactic orientation. (a) Y2H results show interactions between PixE (upper panel) or truncated versions of PixE (lower panel) with PilB1 or the N-terminal cytoplasmic domain of PilC (PilC_{N-term}). For auxotrophic selection, diploid cells harboring prey and bait vectors were grown on restrictive growth medium lacking tryptophan (–Trp) and leucine (–Leu). To test specific interaction, plates additionally lacked histidine (–His) and were supplemented with 3-AT. Empty prey and bait vectors are included as controls. (b) Y2H assays using full-length PixE, the PixE PATAN domain, and the separated N-terminal (residues 1–366) and C-terminal (367–672) domains of PilB1. (c) Fluorescence microscopy images show the subcellular localization of the PixE PATAN domain harboring a C-terminal eYFP fusion. The expression vector and conditions are identical to the strain expressing full-length PixE-eYFP shown in Figure 2. eYFP fluorescence is shown in green and chlorophyll fluorescence in magenta. Scale bars = 5 μm. (d) Phototaxis assays with *Synechocystis* cells harboring a plasmid for the inducible expression of the PixE PATAN domain (PixE_(PATAN)⁺) or a control plasmid (control). Cells were spotted onto 0.5% (w/v) BG-11 motility agar plates and placed in directional white light (~30 μmol photons/m²/s, indicated by the flash symbol) for 11 days. Protein expression was either repressed with 2.5 μM CuSO₄ or induced by copper depletion. (e) Phototaxis assays with *Synechocystis* wild-type cells, a mutant with a *pixG-pixH* deletion inactivating the Tax1 system (Δ *pixGH*) and complementation strains that encode wild-type *pixH* and either the wild-type *pixG* sequence (PixG_(WT)) or truncated versions of PixG (PixG_(REC) and PixG_(PATAN)⁺ or a variant with a phosphor-acceptor site mutation (PixG_(D326A)).

Figure 4c show a fluorescence distribution along the cell periphery comparable with the full-length protein (see Figure 2a for comparison), verifying that the N-terminus is sufficient for membrane localization. In order to test whether membrane localization of PatA-type response regulators depends on PilB1 or PilC, we inactivated the genes in *Synechocystis* strains expressing PixG, PilG, TaxP2, and LsiR eYFP-fusion proteins. However, membrane localization of PatA-type response regulators is not lost in $\Delta pilB1$ or $\Delta pilC$ mutants (Figure S5).

Since the Y2H data suggest that the PATAN domain can interact with pilus components independently of other domains, we sought to characterize the role of this interaction during phototaxis in *Synechocystis*. First, we tested phototactic motility in a *Synechocystis* strain transformed with a plasmid encoding the truncated PixE protein under the control of the copper-repressed *PpetJ* promoter (PixE_{PATAN}⁺). When the ectopic expression of PixE_{PATAN} was repressed, the strain showed positive phototaxis toward white light and was indistinguishable from a *Synechocystis* control strain harboring a control plasmid (Figure 4d). In stark contrast, PixE_{PATAN}⁺ but not the control strain reversed its phototactic orientation and moved away from the light source under conditions that induce protein expression. These experiments imply that PixE_{PATAN} itself can bind to the TFP motor also in *Synechocystis* cells and clearly demonstrate that the PixE PATAN domain is sufficient to act as an output signal. In a similar experiment, we analyzed phototaxis in $\Delta pixGH$ -mutant strains complemented with *pixH* and either the wild-type *pixG* (PixG_{WT}), the truncated variants PixG_{PATAN} or PixG_{REC}, or a mutant missing the conserved aspartate at the putative phosphorylation site (PixG_{D326A}). These motility assays (Figure 4e) demonstrate that only the expression of the full-length PixG with an intact phosphorylation site restored positive phototaxis. This supports the idea that the PatA-type response regulators that are part of chemotaxis operons rely on phosphorylation to switch to an active signaling state.

3 | DISCUSSION

In *Synechocystis*, action spectra of positive and negative phototaxis overlap with the absorption spectra of photosynthesis (Ng et al., 2003), and photoreceptor mutants typically reverse orientation but maintain directional movement in regard to the light vector (Schuergers et al., 2017). Therefore, it can be reasonably assumed that phototaxis in *Synechocystis* is controlled by two distinct regulatory pathways. The first regulatory circuit establishes cell polarity, presumably through indirect light sensing for example, via alterations in proton motive force (Harwood et al., 2021). At the same time, another system integrates the spectral quality of the incoming light to determine the orientation of polarity and thereby the direction of phototaxis. It is apparently the photoreceptors PixJ1, PixD, and UirS that collectively modulate the decision between positive and negative phototaxis via their cognate PatA-type response regulators. Here we could demonstrate that in *Synechocystis*, the PATAN domain of PatA-type response regulators interacts with the TFP extension motor and that the expression of the PixE PATAN domain alone is

sufficient to reverse phototactic orientation. We thus assume that modulation of pilus assembly by the PATAN domain constitutes the principal output of PatA-type regulators in cyanobacteria.

There is direct evidence for an interaction between the cytoplasmic NTD of the platform protein PilC and the motor ATPase PilB (Takhar et al., 2013), and it was suggested that a PilC NTD dimer would fit into the pore formed by a PilB hexamer (McCallum et al., 2017). Given that the PatA regulators interact with both the cytoplasmic NTD of PilC and the N-terminal domain of PilB1, we speculate that the PATAN domain modulates this interaction between the extension motor and the pilus platform. The PATAN domains found in *Synechocystis* and many other bacteria contain a small insertion of two repetitive units, each forming two antiparallel α -helices. Interestingly, repeats of this α -clip domain are also frequently found at the start of the N1 domain of various type II secretion systems (T2SS) and TFP extension ATPases (Makarova et al., 2006). This N1 domain is thought to play a key role in engaging inner membrane components of the TFP machinery (Collins et al., 2018). Therefore, it is conceivable that the PATAN domain might share structural similarities with the N-terminus of PilB and that both proteins share—or compete for—a common interaction partner. While we do not know if the self-interaction observed for most PatA-type regulators has any regulatory consequence, we could imagine that this multimer formation might be relevant when many regulators bind the hexameric PilB1 simultaneously.

The REC domain, on the other hand, is likely required to convey sensory input from the MCP through phosphorylation by its cognate histidine kinase. So far, there is no direct evidence of phosphotransfer between CheA homologs and PatA regulators based on experimental data. In vitro phosphotransfer assays showed that the PatA-type regulator HmpA from *N. punctiforme* does not accept phosphate from the Hmp system (Risser et al., 2014). However, we observed that the PixG(D326A) variant fails to complement the mutant phenotype (Figure 4e). Together with other circumstantial evidence like the interaction between PilG and the histidine kinase PilL in *Synechocystis* (Kera et al., 2020), this supports the idea that phosphotransfer is essential for the function of at least some PatA-type regulators from chemosensory systems. The orphan regulators, on the other hand, seem to be mostly phosphorylation independent because they lack conserved residues required for phosphorylation (Figure S2). The reversal of phototactic orientation in *Synechocystis* expressing only the PATAN domain of PixE (Figure 4d) and the successful complementation of an *Anabaena patA* mutant by a truncated gene missing the REC domain (Young-Robbins et al., 2010) indicate that, in general, the PATAN domain can exert its function independently of other domains.

While we still do not know how the interplay between CheY-type and PatA-type regulators affects the TFP motor, a comparison with chemotactic bacteria suggests a possible mode of action. The disparate localization patterns of the chemosensory response regulators PixG/PixH and PilG/PilH (Figure 2a) are reminiscent of the chemosensory Chp system that regulates twitching migration in *Pseudomonas aeruginosa* (*P. aeruginosa*) (Bertrand et al., 2010).

P. aeruginosa PilG, which does not harbor a PATAN domain, localizes to the cell pole where it promotes polar recruitment of the extension motor PilB favoring forward movement. PilH, which is diffusely localized in the cytoplasm, counteracts unipolar PilB activity, thereby facilitating reversals of cell movement (Kühn et al., 2021). We envision that the response regulators of cyanobacterial Types 2–4 chemotaxis systems follow a similar division of labor with the PatA-type regulators promoting unipolar PilB1 activity by directly binding to the TFP motor. PilG, the PATAN-domain regulator of the Type 1 chemotaxis system did not show clear interaction with PilB1 or PilC in our study. Considering that *Synechocystis* PilG and the orthologous HmpA from *N. punctiforme* are dispensable for phototactic motility (Risser et al., 2014; Yoshihara et al., 2002), their mechanistic function might differ from that of the other PATAN-domain regulators. Interestingly, the cyanobacterial Type 1 chemotaxis systems (Figure 1a) are linked to the cyanobacteria-specific HmpF protein, a filament-forming protein essential for motility in *Synechocystis* and *N. punctiforme*, that exhibits dynamic, unipolar localization to the leading cell poles in motile hormogonia (Bhaya et al., 2001b; Cho et al., 2017; Harwood et al., 2021; Springstein et al., 2020). This additional key component steers cell polarity and positive phototaxis in *N. punctiforme* by interaction with the pilus motor via Hfq and PilT (Harwood et al., 2021). It is suggested that the widely conserved Type 1 chemotaxis systems control phototaxis via HmpF by sensing and responding to a change in the proton motive force in many cyanobacteria including *Synechocystis* (Harwood et al., 2021).

PixE has been shown to co-localize with PilB1 (Jakob et al., 2019) and has been identified in protein complexes up to ~450 kDa in membrane fractions separated by native PAGE (Sugimoto & Masuda, 2020). The truncated PixE PATAN domain localizes to the membrane (Figure 4c) indicating that it determines subcellular localization. Nonetheless, membrane localization of different PatA-type regulators is not lost by the deletion of the *pilB1* or *pilC* genes, suggesting that other binding partners exist. This hypothesis is based on data from *P. aeruginosa* which show that membrane localization of PilB is lost in a *pilC* mutant (Chiang et al., 2005) and our observation that the function of Hfq, a binding partner of PilB1 depends on PilC (Schuergers et al., 2014). However, we can not exclude that PilB is able to localize to the membrane even in the absence of PilC, as it was shown for *Myxococcus xanthus* (Friedrich et al., 2014). In this case, PilB1 could still recruit the PatA-type response regulators to the membrane and no additional binding partners would be requested.

Several studies in *Synechocystis* and other cyanobacteria indicate that a variety of proteins that are linked to TFP function can interact with the pilus base, among them EbsA (Yegorov et al., 2021), Hfq (Schuergers et al., 2014), Slr0845 (Schuergers & Wilde, 2015), and the aforementioned HmpF (Harwood et al., 2021; Springstein et al., 2020). Hence, one must assume that the pilus motor complex is a protein interaction hub where distinct protein subassemblies regulate TFP with various functions. Therefore, we speculate that apart from PilB1 and PilC, *Synechocystis* PatA-type regulators

interact with other proteins to determine the orientation of pilus assembly during phototaxis.

In contrast to the PatA-type regulators in *Synechocystis*, the orphan regulator PatA localizes to the polar regions of heterocysts or the central plane of dividing cells when overexpressed in *Anabaena* (Valladares et al., 2020; Young-Robbins et al., 2010). An interaction with the TFP motor during nitrogen starvation or cell division is not immediately plausible. These findings corroborate that at least some orphan PatA-type regulators evolved specific functions other than controlling TFP assembly. Nonetheless, the distinct localization patterns of PatA support the idea that this orphan regulator likewise regulates spatiotemporal protein dynamics. In light of these findings, we argue that a predicted role of cyanobacterial PatA-type regulators as DNA-binding proteins (Makarova et al., 2006) seems unlikely.

PatA-type response regulators are typical for cyanobacteria, whereas PATAN-containing proteins of variable domain architectures are widespread in other environmental bacteria. Many genomes encode multiple PATAN domain-containing proteins (IPR025497/DUF4388), with some species like *M. xanthus* having more than a dozen. While the majority of the more than 8000 proteins containing a DUF4388 annotation comprise no additional domains or harbor a single REC domain, other proteins contain a variety of different domains. Remarkably, there is a number of proteins that are annotated as containing a GspE_N domain (PF05157) which is prototypical for the N-terminus of PilB1. This co-occurrence might hint at an evolutionarily conserved association between the PATAN domain and T2SS assembly ATPases. What little is known from PATAN domain proteins outside the cyanobacteria also hints at a connection between this domain and the TFP apparatus. In *M. xanthus*, the PATAN-domain protein SgnC is required for social gliding motility (Yoderian et al., 2003). Furthermore, homologs of MglA and MglB that control asymmetrical assembly of TFP by sorting PilB and PilT to opposite cell poles are regularly encoded in the genetic neighborhood to PATAN-containing proteins (Wuichet & Søgaard-Andersen, 2014). It remains to be seen if there is a conserved role of PATAN domains in the spatiotemporal regulation of TFP machineries.

4 | MATERIALS AND METHODS

4.1 | Phylogenetic and sequence analysis

PatA-type response regulators in indicated genomes were detected with hmmsearch using an HMM model generated from an alignment of the six PATAN-containing regulators from *Synechocystis*. Protein sequences were aligned with MAFFT and subsequently refined using Guidance2 (Sela et al., 2015) with the following settings (bootstraps 100; maxiterate 1000, local pair). Unreliable columns with GUIDANCE scores below 0.93 were discarded. The maximum-likelihood phylogenetic tree was inferred using an LG model with a discrete gamma distribution (+G). The

percentages at nodes are bootstrap probabilities calculated using 500 replicates. We considered phosphorylation of REC domains unlikely when either the second aspartate of the conserved pair following β -sheet 1, the aspartate of the phosphorylation site, or the conserved threonine/serine at the end of β -sheet 4 was missing (Bourret, 2010).

4.2 | Culture condition and strains

The glucose-tolerant motile *Synechocystis* sp. PCC 6803 PCC-M wild type and mutants (Table S1) were propagated on BG11 agar plates (0.75% (w/v)) supplemented with 0.3% (w/v) sodium thiosulfate. Liquid cultures were grown in BG11 medium supplemented with 10 mM N-[Tris-(hydroxymethyl)-methyl]-2-aminoethanesulphonic acid (TES) buffer (pH 8.0) at 30°C under continuous white-light illumination (Philips TLD Super 80/840) of 50 $\mu\text{mol photons/m}^2/\text{s}$ and constant shaking. For induction of the *PpetJ* promoter, CuSO_4 was omitted from the medium. For mutant strains, antibiotics were added at the following concentrations: chloramphenicol, 7 $\mu\text{g/ml}$; streptomycin, 10 $\mu\text{g/ml}$; kanamycin, 40 $\mu\text{g/ml}$; zeocin, 5 $\mu\text{g/ml}$, and gentamycin, 10 $\mu\text{g/ml}$.

4.3 | Mutagenesis and plasmid construction

All primers and plasmids are listed in Tables S1 and S2. *Synechocystis* strains expressing the PatA-type response regulators with a C-terminal eYFP tag under the control of a copper-sensitive *petJ*-promoter were constructed as follows: The *Synechocystis* genes *pixG* (slr0038), *pixH* (slr0039), *pilG* (slr1041), *pilH* (slr1042), *IsiR* (slr1214), and *pixE* (slr1693) were amplified from genomic DNA using primers to introduce NdeI and XhoI restriction sites. Each fragment was subsequently cut and ligated into the NdeI and XhoI restriction site of the pSK-hfq-eyfp vector harboring a C-terminal eYFP (Schuergers et al., 2014). The resulting plasmids were verified by sequencing and used to transform wild-type *Synechocystis*, leading to the integration of the expression cassette into a neutral locus via homologous recombination (Kuchmina et al., 2012). Furthermore, these strains were transformed with genomic DNA from mutant strains to inactivate the *pilB1* or *pilC* genes (Schuergers et al., 2014). For inducible expression in $\text{PixE}_{(\text{PATAN})}^+$ the PixE PATAN domain was amplified from genomic DNA using primers to introduce NdeI and BglII restriction sites and subsequently cut and ligated into the appropriate restriction site of a pUR-expression vector harboring a C-terminal FLAG sequence (pUR-C-FLAG). For the deletion and subsequent complementation of the *pixGH* genes *Synechocystis* cells were transformed with plasmids pUC- ΔpixGH and pUC-*pixG*(WT)H, which were constructed by seamless cloning. Variants containing a mutated *pixG* gene were constructed by oligonucleotide-directed mutagenesis of pUC-*pixG*(WT)H. Complete segregation of these mutants was verified by colony PCR.

4.4 | Phototaxis assays

The phototactic movement was analyzed as described previously (Jakob et al., 2017). In short, cell suspensions were spotted on 0.5% (w/v) BG11 agar plates supplemented with 0.2% glucose and 10 mM TES buffer (pH 8.0). For repression of the *petJ* promoter 2.5 μM CuSO_4 was added. After 2–3 days under diffuse white light (50 $\mu\text{mol photons/m}^2/\text{s}$) the plates were placed into nontransparent boxes with a one-sided opening ($>5 \mu\text{mol photons/m}^2/\text{s}$) for at least 10 days.

4.5 | Y2H analysis

The Y2H analysis was carried out as described previously (Jakob et al., 2019). Primers for the construction of yeast expression plasmids are listed in Table S1. *Synechocystis* DNA fragments were amplified from genomic DNA, subsequently cut with the appropriate restriction enzymes, and ligated into the appropriately cut pCGAD-T7ah, pGADT7ah, pD153, or pGBKT7 bait and prey vectors, creating either N- or C-terminally tagged GAL4 activation domain (AD/prey) or GAL4 DNA-binding domain (BD/bait) fusion proteins. Yeast transformants containing bait-prey pairs were selected on a complete supplement mixture (CSM) dropout medium lacking leucine and tryptophan at 30°C for 4 days. Subsequently, cells were screened for interaction by streaking on a dropout medium lacking leucine, tryptophan, and histidine supplemented with different amounts of 3-amino-1,2,4-triazole (3-AT) at 30°C for 6–7 days.

4.6 | Epifluorescence microscopy

Micrographs were captured at room temperature using an upright Nikon Eclipse Ni-U microscope fitted with an x40 (numerical aperture 0.75) and an x100 oil immersion objective (NA 1.45). For visualization of epifluorescence, a GFP-filter block (excitation 450–490 nm; emission 500–550 nm; exposure time 1 s) or a Cy3-filter block (excitation 530–560 nm; emission 575–645 nm; exposure time <50 ms) were used. Cells were picked from colonies grown on phototaxis plates, resuspended in a small volume of PBS pH 7.4 buffer, and spotted on glass slides. Fluorescence distribution was analyzed with ImageJ (1.53c) using the Radial Profile Extended plugin and normalization and curve fitting (LOESS) was calculated using custom R code.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Y.H., N.S., and A.W. designed the study and wrote the manuscript. Y.H., A.J., S.E., and N.S. performed and analyzed the experiments. All authors interpreted and discussed the data.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supplementary Material of this article.

ORCID

Annegret Wilde  <https://orcid.org/0000-0003-0935-8415>

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