Novel Photosensory Two-Component System (PixA–NixB–NixC) Involved in the Regulation of Positive and Negative Phototaxis of Cyanobacterium Synechocystis sp. PCC 6803

Rei Narikawa1,2,*, Fumiko Suzuki1, Shizue Yoshihara3, Sho-ichi Higashi4, Masakatsu Watanabe5 and Masahiko Ikeuchi1

1Department of Life Sciences (Biology), Graduate School of Art and Sciences, University of Tokyo, Komaba 3-8-1, Meguro, Tokyo, 153-8902 Japan
2Japan Science and Technology Agency (JST), PRESTO, 4-1-8 Honcho Kawaguchi, Saitama, 332-0012 Japan
3Department of Biological Science, Graduate School of Science, Osaka Prefecture University, Gakuen-cho 1-1, Naka-ku Sakai, Osaka, 599-8531 Japan
4Spectrography and Bioimaging Facility, NIBB Core Research Facilities, National Institute for Basic Biology, Okazaki, Aichi, Japan
5The Graduate School for the Creation of New Photonics Industries, Kurematsu-cho 1955-1, Nishi-ku, Hamamatsu, Shizuoka, 431-1202 Japan

*Corresponding author: E-mail, narikawa@bio.c.u-tokyo.ac.jp; Fax, +81-3-5454-4337.
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Two wild-type substrains of a motile cyanobacterium Synechocystis sp. PCC 6803 show positive phototaxis toward a light source (PCC-P) and negative phototaxis away from light (PCC-N). In this study, we found that a novel two-component system of photoreponse is involved in the phototactic regulation. Inactivation of slr1212 (pixA), which encodes a photoreceptor histidine kinase, reverted the positive phototaxis of PCC-P to negative phototaxis, and inactivation of the downstream slr1213 (nixB) and slr1214 (nixC), which encode AraC-like transcription factor-type and PatA-type response regulators, respectively, reverted the negative phototaxis of PCC-N to positive phototaxis. Opposite effects of pixA and nixBC disruption implies an unexpected signal transduction pathway in the switching of positive and negative phototaxis. The blue/green-type cyanobacteriochrome GAF domain of PixA was expressed in Synechocystis and phycocyanobilin-producing Escherichia coli. The holoprotein covalently bound a chromophore phycoviolobilin and showed reversible photoconversion between the violet- (Pv, \( \lambda_{\text{peak}} = 396 \) nm) and green-absorbing (Pg, \( \lambda_{\text{peak}} = 533 \) nm) forms, although the protein from E. coli partially bound a precursor phycocyanobilin. These results were discussed with regard to an idea that PixA serves as a violet light receptor for switching of positive and negative phototaxis by transcriptional and functional regulation.

Keywords: Cyanobacteriochrome • Phototaxis • Two-component system • Violet light.

Abbreviations: CBB, Coomassie brilliant blue R-250; LED, light-emitting diode; PCB, phycocyanobilin; Pg, green-absorbing form; Pv, violet-absorbing form; PVB, phycoviolobilin.

Introduction

Sensing light is important for many organisms, especially for phototrophnic organisms, since they largely depend on light energy, which is ubiquitous but variable in natural environments on the Earth. One of the typical photoreceptors is the plant, fungal and bacterial phytochrome that binds linear tetrapyrrole and show reversible photoconversion between red- and far-red-absorbing forms. Recently, cyanobacteriochromes that are related to but clearly distinct from the phytochromes have been identified. They showed unique and diverse photo-chemical properties (Ikeuchi and Ishizuka 2008); blue/green reversible photoconversion of SyPixJ1/TePixJ and Tlr0924 (Yoshihara et al. 2004, Ishizuka et al. 2006, Rockwell et al. 2008), green/red reversible photoconversion of AnPixJ and CcaS (Hirose et al. 2008, Narikawa et al. 2008a, Hirose et al. 2010) and unidirectional photoconversion from the violet-absorbing form to the yellow-absorbing form and slight dark reversion of SyCikA (Narikawa et al. 2008b). The blue/green and green/red photo-reversible cyanobacteriochromes bound phycoviolobilin (PVB) and phycocyanobilin (PCB) chromophores, respectively. Many other candidates for the cyanobacteriochrome still remain to be characterized.
Cell motility of many cyanobacteria has been shown as gliding, swimming or twitching in the absence of flagella (Diehn et al. 1977, Waterbury et al. 1985, Haeder 1987). Some cyanobacteria show a directional movement of cells toward the light source (positive phototaxis) or a movement away from the light source (negative phototaxis) (Stanier et al. 1971, Nultsch et al. 1979). Mutational analyses have revealed that a unicellular motile cyanobacterium *Synechocystis* sp. PCC 6803 showed twitching motility on solid surfaces using a type IV pilus structure (Bhaya et al. 2000, Yoshihara et al. 2001). Further studies have identified genes involved in phototaxis: genes in the *pix*1 cluster (or *taxD1*), *pixD* and *cph2* (Bhaya et al. 2000, Yoshihara et al. 2000, Bhaya et al. 2001, Wilde et al. 2002, Okajima et al. 2005). Positive phototaxis of the wild type was lost or affected in these mutants. Mostly, they showed negative phototactic motility. Even the wild type that shows positive phototaxis under normal light conditions is able to show negative phototaxis under high light conditions (Ng et al. 2003). Moreover, a distinct substrain of the wild type that shows negative phototaxis under normal light conditions was also isolated from *Synechocystis* sp. PCC 6803 (Yoshihara et al. 2000). It is thus suggested that the phototactic motility of wild-type *Synechocystis* is regulated on the balance between positive and negative phototactic potentials.

Of the genes essential for positive phototaxis, *pix*1 (or *taxD1*) and *pixD* have been shown to encode photoreceptor proteins. *Pix*1 binds PVB and shows reversible photoconversion between blue- (Pb) and green-absorbing (Pg) forms (Yoshihara et al. 2004, Ikeuchi and Ishizuka 2008). *Pix*D binds a flavin and shows a light-induced red shift of the flavin absorption as a typical BLUF protein (Okajima et al. 2005). These proteins seem to serve as a blue light sensor that may switch off the positive phototaxis, allowing the emergence of negative phototaxis. Moreover, it has been recently reported that *Cph2* is a negative regulator in phototaxis toward UV-A and acts upstream of *Pix*1 (Moon et al. 2011). However, the molecular mechanism of the regulation of phototaxis still remains largely unknown.

In this communication, we identified a novel photoreceptor histidine kinase gene, *slr1212* (*pixA*) essential for positive phototaxis and novel negative phototaxis-related genes, *slr1213* (*nixB*) and *slr1214* (*nixC*), which are located downstream of *pixA*, to encode potential two-component response regulator proteins. We further reported the spectral properties of *Pix*A, suggesting a novel violet light sensor.

**Results**

**Phototaxis assay**

The phototactic motility of *Synechocystis* mutants defective in *slr1212*, *slr1213* and *slr1214* genes was evaluated as the movement of colonies on a soft agar plate under lateral illumination. The PCC-P and PCC-N substrains used for gene disruption reproducibly moved toward the light source (positive phototaxis) and away from the light source (negative phototaxis), respectively (Yoshihara et al. 2000). The *slr1212*-disruptant of PCC-P strain showed negative phototaxis away from the light source, while that of the PCC-N strain showed negative phototaxis like the parent strain (Fig. 1). On the other hand, the *slr1213*- and *slr1214*-disruptants of the PCC-N strain showed positive phototaxis toward the light source, while those of the PCC-P strain showed positive phototaxis like the parent strain. We confirmed that all independent transformants tested showed the same phenotype. These results suggest that *slr1212* encodes a factor for positive phototaxis, whereas *slr1213* and *slr1214* encode factors for negative phototaxis. So we designated *slr1212*, *slr1213* and *slr1214* as *pixA*, *nixB* and *nixC*, respectively.

**Wavelength dependence of phototactic motility**

To gain insights into the photosensory mechanism, the wavelength dependence of phototaxis was examined for the PCC-P substrain and the *pixA* mutant on the PCC-P background (Fig. 2). The PCC-P substrain showed positive phototaxis toward the light source from 510 to 760 nm. On the other hand, the *pixA* mutant showed negative phototaxis away from the light source from 510 to 710 nm.

**Domain architecture of PixA, NixB and NixC**

*Pix*A harbors three transmembrane regions, two PAS (Per/ARNT/Sim), one GAF (GMP-phosphodiesterase/adenylate cyclase/FhlA) and one His kinase (HK) domain (Fig. 3A). The N-terminal transmembrane region has been reported to bind ethylene directly (Rodriguez et al. 1999). The GAF domain of *Pix*A is another reponse regulator called PatA-type (Liang et al. 2000, Bhaya et al. 2000, Yoshihara et al. 2001). Further studies showed the same phenotype. These results suggest that *slr1212* encodes a factor for positive phototaxis, whereas *slr1213* and *slr1214* encode factors for negative phototaxis. We confirmed that all independent transformants tested showed the same phenotype. These results suggest that *slr1212* encodes a factor for positive phototaxis, whereas *slr1213* and *slr1214* encode factors for negative phototaxis. So we designated *slr1212*, *slr1213* and *slr1214* as *pixA*, *nixB* and *nixC*, respectively.

**His-tagged PixA-GAF purified from Synechocystis**

The recombinant His-tagged GAF domain of *Pix*A (*Pix*A-GAF) was expressed in *Synechocystis* and purified by nickel affinity chromatography. The purified fractions were concentrated by ultrafiltration and subjected to SDS–PAGE. Although the purified fraction contains many contaminated proteins probably due to low expression level of *Pix*A-GAF, a CBB (Coomassie brilliant blue R-250)-stained band clearly corresponded to the zinc-induced fluorescent band at the predicted molecular size of 23 kDa (Supplementary Fig. S2, Fig. 3B). After SDS–PAGE, proteins were transferred to a membrane and subjected to Western

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*Note:* The text is a natural representation of the document's content as provided. Further contextual information may be required for full understanding.
blotting analysis with HisProbe. As a result, a fluorescent band specifically reacted with HisProbe (Fig. 3B). These results suggest that PixA-GAF covalently binds a linear tetrapyrrole chromophore in vivo.

The PixA-GAF isolated under room light was a mixture of two spectral forms. Irradiation of purified PixA-GAF with a green light yielded a prominent peak at 396 nm at the cost of a peak in a green region (Fig. 4A). When this sample was next irradiated with a violet light, the peak in the violet region completely disappeared and a peak at 533 nm and a second minor peak around 330 nm appeared concomitantly. We also detected a peak around 620 nm perhaps derived from contaminated phycocyanin. In conclusion, PixA-GAF isolated from Synechocystis showed reversible photoconversion between the violet-absorbing form (Pv) and the green-absorbing form (Pg). The absorption difference spectrum of the Pg minus the Pv form showed two positive peaks at 530 and 332 nm and a negative peak at 399 nm (Fig. 4C). The 332 nm peak corresponds to the second peak of the Pg form.

**His-tagged PixA-GAF purified from PCB-producing Escherichia coli**

PixA-GAF was expressed in PCB-producing *E. coli* and purified to homogeneity by nickel affinity chromatography (Supplementary Fig. S3, Fig. 3C). The band corresponding to PixA-GAF showed intense fluorescence in the presence of zinc ions, indicative of covalent binding with a linear tetrapyrrole chromophore (Fig. 3C). Like the preparation isolated from *Synechocystis*, PixA-GAF isolated from PCB-producing *E. coli* showed reversible photoconversion between Pv ($\lambda_{\text{peak}} = 396$ nm, Figs. 3D, 4B) and Pg forms ($\lambda_{\text{peak}} = 533$ nm,
Fig. 2 Wavelength dependence of the PCC-P substrain and the pixA mutant on the PCC-P background. Distance represents migration of the edge of the colony. The fluence rate of actinic light was 0.7 μmol m⁻² s⁻¹. Similar results were obtained in three independent experiments.

Fig. 3 Domain architecture and purification. (A) Domain architecture of PixA. The black bar indicates the expressed region of PixA-GAF. (B) SDS–PAGE of PixA-GAF isolated from Synechocystis. CBB staining (CBB) and fluorescence detection with (+Zn) or without zinc ion (−Zn) and Western blotting with HisProbe (Western). (C) SDS–PAGE of PixA-GAF isolated from PCB-producing E. coli. CBB staining (CBB) and fluorescence detection with (+Zn) or without zinc ion (−Zn). (D) Photographs of the Pv and Pg forms of PixA-GAF solution isolated from PCB-producing E. coli.

Fig. 4 Absorption and difference spectra of native PixA-GAF. (A) Pv (broken line) and Pg (solid line) forms of PixA-GAF isolated from Synechocystis. (B) Pv (broken line) and Pg (solid line) forms of PixA-GAF isolated from PCB-producing E. coli. (C) Pg minus Pv difference spectra of PixA-GAF isolated from Synechocystis and PCB-producing E. coli. The difference spectrum of the E. coli-expressed protein had a shoulder at around 620 nm (arrowhead).
Figs. 3D, 4B). Such photoconversion can be repeated many times without appreciable deterioration. Although these spectral properties are similar to those of PixJs of *Synechocystis* and *Thermosynechococcus elongatus*, the peak position of PixA Pv is significantly blue-shifted in comparison with those of Pix Pb forms (430 nm) (Yoshihara et al. 2004, Ishizuka et al. 2006). The absorption difference spectrum of Pg minus Pv is almost identical to that isolated from *Synechocystis* (Fig. 4C). These spectra showed two positive peaks at 530 and 332 nm and a negative peak at 399 nm. The 332 nm peak corresponds to the second peak of the Pg form. However, it should be noted that the difference spectrum of the *E. coli*-expressed protein had a shoulder at around 620 nm, which was not detected in that of the *Synechocystis*-expressed protein. This difference may be derived from the different chromophore composition between these two preparations as described below.

### Denaturation with acidic urea

To gain insights into chromophore species and configuration, we denatured PixA-GAF purified from *Synechocystis* and PCB-producing *E. coli* under acidic conditions. Denatured spectra of the Pv and Pg forms of PixA-GAF purified from *Synechocystis* had broad peaks at around 550–600 and 500–550 nm, respectively, although their spectra were very noisy due to low yield (Fig. 5A). The difference spectrum of the denatured Pv minus denatured Pg of PixA-GAF had a positive peak at around 600 nm and a negative peak at around 515 nm (Fig. 5C). These spectral properties roughly corresponded to those of TePixJ isolated from *Synechocystis* (Ishizuka et al. 2007), suggesting that the native chromophore of PixA is PVB.

On the other hand, denatured spectra of the Pv and Pg forms of PixA-GAF purified from PCB-producing *E. coli* had peaks at 596 and 550 nm, respectively (Fig. 5B), and the difference spectrum had positive peaks at 602 and 667 nm and a negative peak at around 515 nm (Fig. 5C). These spectral properties roughly corresponded to those of C15-Z isomers of PCB and PVB (Ishizuka et al. 2007), we can assign the 602 and 667 nm peaks to PVB and PCB, respectively. This assignment was further confirmed by photoconversion of the denatured chromophore with white light (Fig. 6). The denatured chromophore derived from Pg was largely photoconverted, while that derived from Pv was slightly photoconverted (Fig. 6A, B). The difference spectrum of the photoconversion of the denatured Pg showed double peaks at 602 and 667 nm, whereas that of the denatured Pv showed a single peak at 602 nm (Fig. 6C). This fits well with the fact that C15-E PCB is unidirectionally photoconverted to C15-Z, while both C15-E and C15-Z of PVB are photoconverted to equilibrium (Ishizuka et al. 2007). Thus, the *E. coli*-expressed PixA-GAF harbored a small but significant amount of PCB, in addition to PVB as a major chromophore. In contrast, the *Synechocystis*-expressed PixA-GAF consisted of PVB (Fig. 5C). Since PCB is supplied in *E. coli*, efficient but incomplete isomerization from PCB to PVB proceeded in the PixA-GAF protein. Violet/green reversible

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**Fig. 5** Absorption and difference spectra of denatured PixA-GAF with acidic urea. (A) Pv (broken line) and Pg (solid line) forms of PixA-GAF isolated from *Synechocystis*. (B) Pv (broken line) and Pg (solid line) forms of PixA-GAF isolated from PCB-producing *E. coli*. (C) Pv minus Pg difference spectra of PixA-GAF isolated from *Synechocystis* and PCB-producing *E. coli*.
photoconversion, but no details, was reported for in vitro reconstituted PixA-GAF with PCB (Ulijasz et al. 2009).

**Discussion**

In this communication, we identified a photoreceptor gene (pixA) for positive phototaxis and regulator genes (nixB and nixC) for negative phototaxis. The GAF domain of PixA purified from Synechocystis and PCB-producing E. coli cells showed similar reversible photoconversion between the Pv (396 nm) and Pg (533 nm) forms, although differences were observed in the difference spectrum and chromophore composition. Importantly, the Pv form was blue-shifted by >30 nm in comparison with the Pb form of the blue light sensor TePixJ (430 nm), implying that PixA is a novel violet light sensor.

It has been shown that the blue/green and green/red photoreversible cyanobacteriochromes bound PVB and PCB, respectively (Ikeuchi and Ishizuka 2008). Sequence comparison showed that the blue/green-type cyanobacteriochromes possess a second conserved cysteine residue as well as the canonical cysteine residue, while the green/red-type cyanobacteriochrome conserved only the canonical cysteine residue. Site-directed mutagenesis revealed that the second cysteine residue is essential for photoactive blue/green holoprotein (Rockwell et al. 2008, Ulijasz et al. 2009, Ishizuka et al. 2011). Fourier transform infrared (FTIR) measurement suggested that the second cysteine residue transiently forms a covalent bond with C10 of PVB to enable it to absorb the blue light (Ishizuka et al. 2011). On the other hand, Ulijasz et al. (2009) reported that the chromophore is stably ligated with both the canonical and second cysteine residues. There is still an argument about the photoconversion mechanism of the second cysteine-type cyanobacteriochrome. Anyway, the violet/green-absorbing PixA-GAF also conserved both canonical and second cysteine residues. Here we examined the sequence feature of PixA by clustering analysis of cyanobacteriochrome GAF domains (Fig. 7). As a result, a subcluster that includes PixA-GAF was found specifically to possess a short insertion and a conserved third cysteine residue, whose positions were rather near the chromophore-binding pocket based on the mapping to a known structure (Supplementary Fig. S4). These features might be crucial for the shorter wavelength absorption of the Pv form. In particular, the third cysteine residue may transiently form an adduct with the chromophore in Pv form. It would be interesting to characterize the other members of this subcluster by expressing them in E. coli and Synechocystis cells.

Here we demonstrated that the violet/green-absorbing PixA is essential for positive phototaxis. Whereas PCC-P showed positive phototaxis toward the light source of the orange–red light region, the pixA mutant on the PCC-P background showed negative phototaxis away from the light source of a similar orange–red region (Fig. 2). These results may suggest that an unidentified orange–red light sensor mainly regulates the
phototactic motility and PixA serves as a kind of master switch. Similar interpretations have been adopted for the other blue light sensors PixJ1 and PixD that show a similar phototactic phenotype (Okajima et al. 2005). Recently, it has been reported that Cph2 is a negative regulator in phototaxis toward UV-A and acts upstream of PixJ1 (Moon et al. 2011). As Cph2 also possesses a second cysteine-type cyanobacteriochrome GAF domain, Cph2 may also be involved in perception of UV to
Thus, NixC is the fourth PatA homolog in Synechocystis why under such light conditions. However, it is still unclear light sensor PixA may well switch off the positive phototaxis the blue light sensors PixJ1 and PixD and the putative violet stresses on the photosystem machinery (Ohnishi et al. 2005), blue light. Since the UV to blue light is well known to place ectively (Rodriguez et al. 1999). PixA may be involved in not only violet–UV light stresses. Further, the N-terminal transmembrane region of PixA has been reported to bind ethylene directly (Rodriguez et al. 1999). PixA may be involved in not only phototaxis but also chemotaxis to ethylene or its derivatives.

pixA (slr1212)–nixB (slr1213)–nixC (slr1214) is a unique gene cluster of a two-component regulatory system that is involved in regulation of phototaxis. Physical interaction between PixA and NixC was suggested by yeast two-hybrid screening (Sato et al. 2007). At the moment, we presume that histidine kinase PixA is activated by light and transfers its phospho group to the response regulators NixB and/or NixC, although intensive trials to detect autophosphorylation of PixA have been unsuccessful (data not shown). NixC is homologous to PatA that was reported as a factor involved in patterning of heterocyst formation in the nitrogen-fixing filamentous cyanobacterium Anabaena sp. PCC 7120 (Liang et al. 1992). Now many PatA homologs are found in the motility/phototaxis-related operons in many cyanobacteria. Of these, the positive phototaxis regulator PixG and the motility regulator PilG have been identified by mutational studies in Synechocystis (Yoshihara et al. 2000, Yoshihara et al. 2001). PixE, a putative phototaxis regulator, was shown to interact directly with the positive phototaxis regulator/phoreceptor PixD in Synechocystis (Okajima et al. 2005). Thus, NixC is the fourth PatA homolog in Synechocystis, which has now been shown to be involved in regulation of negative phototaxis.

As described in the Introduction, it is suggested that the phototactic motility of Synechocystis is regulated by the balance between positive and negative phototactic potentials. Based on this assumption, the phenotype of the pixA disruptant on the PCC-P background was interpreted as suppression of positive phototaxis or enhancement of negative phototaxis. If so, the phenotype of the pixA disruptant on the PCC-N background that shows negative phototaxis to a greater extent than the wild type fits this assumption well (Fig. 1A). Similarly, the phenotypes of the nixB and nixC disruptants on the PCC-N background were interpreted as suppression of negative phototaxis or enhancement of positive phototaxis. If so, the phenotypes of nixB and nixC disruptants on the PCC-P background that show positive phototaxis to a greater extent than the wild type also fit this assumption well. These findings may indicate that PixA and NixB/C regulate the balance of positive and negative phototaxis potentials in a similar way.

Materials and Methods

Culture and growth conditions

Motile strains of the unicellular cyanobacterium Synechocystis sp. PCC 6803 were obtained from the Pasteur Culture Collection, and a clone showing vigorous motility of positive phototaxis (PCC-P) and a clone showing vigorous motility of negative phototaxis (PCC-N) were selected as the parent strains for gene disruption. Synechocystis cells were grown in liquid BG11 medium containing 1% (v/v) CO2 with air-bubbling at 31°C and a light intensity of 50 μEm−2s−1.

For cloning and subcloning of DNA in plasmids, E. coli JM109 was used, while BL21 (DE3) pK7271 (Mukougawa et al. 2006) was used for expression of PixA-GAF in pET28a (Novagen). Cells were grown in Luria–Bertani medium. When required, kanamycin, chloramphenicol or ampicillin was added at 20, 20 or 50 μg/ml, respectively.

Construction of disruptants and assay of phototaxis

Fragments carrying slr1212, slr1213 and slr1214 were amplified by PCR with primers (slr1212, 5′-GACGTGGTGGTAAATCCTA-3′, 5′-CTGAGATTATTGTTTAACTCCTG-3′; slr1213, 5′-CCTGGTACCAAAATCTGATT-3′; 5′-GAGGAAATGCTCAATGCC; and slr1214, 5′-AGGCTTGGATAGCGAGAG-3′, 5′-CCAACCTTAAAACACTGC-3′) using the genomic DNA of Synechocystis and Taq polymerase (Ampli-Taq. Applied Biosystems) and then cloned into the pT7Blue-T vector (Novagen) The coding region of slr1212, slr1213 and slr1214 was interrupted at the AarSIHI, HincII and EcoRV sites, respectively, by insertion of Tn5-derived kanamycin, spectinomycin and kanamycin resistance cassettes, respectively, without a kanamycin resistance cassette. Mutants were generated by transformation of wild-type cells with this DNA and selected on BG1plates containing kanamycin or spectinomycin (Hihara and Ikeuchi 1997). Complete segregation was confirmed by PCR with the same primers as those above (Supplementary Fig. S1). For the phototaxis assay, 1 μl aliquots of a concentrated cell suspension (approximately 2 × 109 cells ml−1) were spotted on to 0.8% (w/v) agar-solidified BG11 supplemented with 0.3% (w/v) sodium thiosulfate, 5 mM glucose and 10 μM DCMU to eliminate the effect of photosynthesis on movement, and then incubated for 48 h under lateral illumination with the white light of 50 μEm2s−1 at 31°C.

Wavelength dependence of phototaxis

Aliquots of 1 μl of concentrated cell suspension (approximately 2 × 109 cells ml−1) were spotted on to 0.8% (w/v) agar-solidified BG11 supplemented with 0.3% (w/v) sodium thiosulfate, 5 mM glucose and 10 μM DCMU. Then, plates were placed on the benchtop of an Okazaki Large Spectrograph and were exposed to monochromic light for 40 h (Watanabe et al. 1982). The wavelength dependence of phototaxis was examined under various wavelengths, 360, 460, 510, 560, 660, 710 and
760 nm at 0.7 μmol m⁻² s⁻¹. After incubation, the path of spotted cells was directed either toward or away from the light source. Migrational distance, i.e. the edge of colonies spread directionally against the light source, was measured to quantify phototactic movement of the PCC-P and the pixA mutant.

Informatics

Homology analysis was performed using the BLAST (NCBI-BLAST, version 2.1-2.2) program running locally or on the Web (non-redundant GenBank and SwissProt in NCBI) (Altschul et al. 1997). Motif analysis was performed by Pfam and SMART searches run locally or on the Web (http://www.sanger.ac.uk/Pfam/, http://smart.embl-heidelberg.de/) (Eddy 1998, Schultz et al. 1998). Multiple sequence alignment and phylogenetic trees were constructed by CLUSTAL_X (Thompson et al. 1997). Phylogenetic trees were drawn using Dendroscope (Huson et al. 2007).

Construction of expression plasmids

A DNA fragment corresponding to PixA-GAF was amplified by PCR with a set of synthetic primers (5’-cccataGGAAATTACCCAGCGTATC-3’, 5’-ggtctACTCAATTGCTGGATGGC-3’) and genomic DNA from Synechocystis sp. PCC 6803. The PCR products were cloned into pT7Blue-T vector (Novagen). After verification of the nucleotide sequence, all inserts were excised from the plasmid with BamHI and NdeI and cloned into the pET28a expression vector (Novagen) and pTCTH2031v vector (Ishizuka et al. 2006). The resulting plasmids were named pET28_PixA-GAF and pTCTH2031v_PixA-GAF.

Expression and purification of His-tagged proteins from Synechocystis cells

A non-motile GT strain of Synechocystis was transformed with pTCTH2031v_PixA-GAF. Cells were cultured at 30°C for 2 weeks in 8 liters of BG11 medium containing chloramphenicol and harvested by centrifugation. The cells were then frozen at −80°C, thawed at 4°C, and resuspended in 50 ml of Buffer A consisting of 20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl and 10% (w/v) glycerol. The cells were broken with three passages through a French Press at 1,500 kg cm⁻². The cell extract was centrifuged at 12,000×g for 20 min to remove cellular debris and then centrifuged at 109,200×g for 60 min at 4°C. Recombinant proteins in the supernatants were purified by nickel affinity chromatography (His-Trap Chelating HP, GE Healthcare). After washing, proteins were eluted using a step gradient of 50, 100 and 200 mM imidazole in Buffer A. Most His-tagged proteins were recovered in the 200 mM imidazole fraction, which was then used for further measurements following the removal of imidazole by dialysis.

SDS–PAGE and zinc-induced fluorescence assay

Purified proteins were solubilized with 2% (w/v) lithium dodecylsulfate, 60 mM dithiothreitol and 60 mM Tris–HCl (pH 8.0), and subjected to SDS–PAGE with a 15% (w/v) polyacrylamide gel, followed by staining with CBB. Alternatively, proteins resolved in the SDS–gel were blotted onto a polyvinylidene difluoride membrane (Immobilon, Millipore) and the His tag was visualized with HisProbe (SuperSignal West HisProbe Kit; Pierce) as instructed by the manufacturer. For the zinc-induced fluorescence assay, the SDS–PAGE was performed with or without 1 mM zinc acetate (Berkelman and Lagarias 1986) and fluorescence was visualized through a 605 nm filter upon excitation at 532 nm (FMBIO II; TAKARA).

Spectroscopic analysis

UV and visible absorption spectra of the proteins were recorded with a spectrophotometer (model U-3100 PC, Shimadzu) at room temperature. Green light was provided by a light-emitting diode (LED) with a peak at 530 nm and a 25 nm half-bandwidth (DG530XDG; Stanley Electric). Violet light was provided by an LED with a peak at 400 nm and a 15 nm half-bandwidth (SDL-SN3CUV-A; Sander). After Pv and Pg forms of PixA-GAF were denatured by 8 M urea (pH 2.0) under dark conditions, absorption spectra were measured. After that, both forms were irradiated with white light for 3 min and absorption spectra were also measured.

Supplementary data

Supplementary data are available at PCP online.

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