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Heterologous complementation systems verify the mosaic distribution of three distinct protoporphyrinogen IX oxidase in the cyanobacterial phylum

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Abstract

The pathways for synthesizing tetrapyrroles, including heme and chlorophyll, are well-conserved among organisms, despite the divergence of several enzymes in these pathways. Protoporphyrinogen IX oxidase (PPOX), which catalyzes the last common step of the heme and chlorophyll biosynthesis pathways, is encoded by three phylogenetically-unrelated genes, *hemY, hemG* and *hemJ*. All three types of homologues are present in the cyanobacterial phylum, showing a mosaic phylogenetic distribution. Moreover, a few cyanobacteria appear to contain two types of PPOX homologues. Among the three types of cyanobacterial PPOX homologues, only a *hemJ* homologue has been experimentally verified for its functionality. An objective of this study is to provide experimental evidence for the functionality of the cyanobacterial PPOX homologues by using two heterologous complementation systems. First, we introduced *hemY* and *hemJ* homologues from *Gloeobacter violaceus* PCC7421*, hemY* homologue from *Trichodesmium erythraeum*, and *hemG* homologue from *Prochlorococcus marinus* MIT9515 into a *ΔhemG* strain of *E. coli*. *hemY* homologues from *G. violaceus* and *T. erythraeum,* and the *hemG* homologue of *P. marinus* complimented the *E. coli* strain. Subsequently, we attempted to replace the endogenous *hemJ* gene of the cyanobacterium *Synechocystis* sp. PCC6803 with the four PPOX homologues mentioned above. Except for *hemG* from *P. marinus,* the other PPOX homologues substituted the function of *hemJ* in *Synechocystis.* These results show that all four homologues encode functional PPOX. The transformation of *Synechocystis* with *G. violaceus hemY* homologue rendered the cells sensitive to an inhibitor of the HemY-type PPOX, acifluorfen, indicating that the *hemY* homologue is sensitive to this inhibitor, while the wild-type *G. violaceus* was tolerant to it, most likely due to the presence of HemJ protein. These results provide an additional level of evidence that *G. violaceus* contains two types of functional PPOX.

Keywords: chlorophyll, complementation assay, cyanobacteria, heme, protoporphyrinogen IX oxidase, tetrapyrrole

Introduction

Tetrapyrroles, such as chlorophyll and heme, play vital roles in electron transfer and redox regulation. Chlorophyll captures light energy and converts it into chemical energy in photosynthetic organisms. Heme serves as a prosthetic group of various proteins involved in electron transfer and/or redox regulation in nearly all organisms (for a review, see Dailey et al. 2017). The main branch of the tetrapyrroles biosynthesis pathway follows the same route in bacteria and eukaryotes, containing almost the same set of intermediates: 5-aminolevulinate, the common precursor, is converted to porphyrin intermediates such as uroporphyrinogen III, coproporphyrinogen III or protoporphyrin IX (Proto IX). The biosynthesis pathway diverges into heme and chlorophyll synthesis at the step of a metal (Fe^{2+} or Mg^{2+}) insertion into Proto IX. In spite of the common intermediates, the enzymes involved in these steps differ between organisms. For example, the oxidation of coproporphyrinogen III can be catalyzed by two phylogenetically unrelated enzymes: one enzyme is monooxygenase-related oxygen-dependent coproporphyrinogen oxidase encoded by *hemF* and the other is an oxygen-sensitive enzyme belonging to the radical-SAM family (Goto et al. 2010), encoded by *hemN*.

Three different types of protoporphyrinogen IX oxidase (PPOX) enzymes have been identified, thus far, for the oxidation of the penultimate step of the common biosynthetic branch of the tetrapyrrole biosynthetic pathway. One type of PPOX is related to a flavodoxin belonging to a FAD-containing oxidase superfamily (Boynton et al. 2009). This type of PPOX is encoded by the *hemG* gene, which is mainly distributed in Gammaproteobacteria (Kobayashi et al. 2014). The second type of PPOX also contains FAD, but it is structurally unrelated to the HemG-type PPOX, with structural similarity to *p*-hydroxybenzoate-hydroxylase (Koch et al. 2004). This type of PPOX is encoded by the *hemY* gene, which has homologues distributed in a wide range of organisms, including all three domains of life (Kobayashi et al. 2014). It is noteworthy that eukaryotes exclusively use this type of PPOX (Kobayashi et al. 2014). The *hemJ* gene encodes the third type of PPOX, which shows a weak phylogenetic similarity to the M subunit of NADH dehydrogenase complex I (Kato et al. 2010). Boynton et al (2011) showed that it requires an additional soluble component for the full PPOX activity (Boynton et al. 2011), which is potentially coproporphyrinogen III oxidase, as it was shown a cyanobacterial *hemJ* mutant accumulates coproporphyrinogen III, the substrate of coproporphyrinogen III oxidase

(Skotnicová et al. 2018). Some organisms code for none of the three PPOX homologues, suggesting there is another yet unidentified PPOX in these organisms (Kato et al. 2010) or that they simply do not synthesize heme or even use another pathway entirely (Kobayashi et al. 2014).

Interestingly, homologues of all three types of PPOX are found in the cyanobacterial phylum. Five marine cyanobacterial species, *Prochlorococcus marinus* (MIT9215 and MIT9515), and *Synechococcus* strains (MIT S9220, MIT S9508, and CPC35) contain *hemG* homologues, while many freshwater and marine cyanobacteria, including *Gloeobacter violaceus* PCC7421 and *Trichodesmium erythraeum*, possess *hemY* homologues (Kobayashi et al. 2014). The remainder of sequenced cyanobacteria species have *hemJ* homologues. A few cyanobacterial species code for more than one type of PPOX homologues. For example, *G. violaceus* contains both a *hemY* and a *hemJ* homologue. Given that each of these homologues encode functional PPOX enzymes in these species, it is intriguing that the PPOX enzyme complement is so divergent within this phylum. This distribution also raises the question of whether these PPOX isoenzymes are all functional and interchangeable between cyanobacterial species. As a first step to answer these questions, we attempted to verify whether all types of PPOX homologues in the cyanobacterial phylum truly encode functional PPOX.

For this purpose, we adopted two heterologous complementation systems (Boynton et al. 2011; Kato et al. 2010). First, we introduced cyanobacterial *hemG*, *hemY* and *hemJ* homologues into a Δ*hemG* mutant of *Escherichia coli* to test if these genes restore the growth of the mutant. In the second complementation system, we attempted to replace the endogenous *hemJ* gene of the cyanobacterium *Synechocystis* sp. PCC6803 (Synechocystis) with the other PPOX homologues. Finally, we tested whether the transformed Synechocystis cells or wild-type *G. violaceus* cells were sensitive to acifluorfen, an inhibitor of the HemY-type PPOX (Jacobs et al. 1990). These experiments verified whether the cells were dependent on the HemY-type PPOX, and revealed that wild-type *G. violaceus* cells could, indeed, grow in the presence of acifluorfen, indicating that the HemJ-type PPOX was sufficient to sustain the growth of cells under laboratory conditions.

Materials and Methods

Bacterial strains and culture conditions

Synechocystis sp. PCC6803 (Synechocystis) and its transformants were cultured in BG-11 medium (Waterbury and Stanier 1981) under continuous illumination (30–50 μmol photons m^{-2} s^{-1}) at 23 ºC. For the growth of transformants, either or both kanamycin and chloramphenicol were added to the media in the final concentrations of 50 μg ml^{-1} or 25 μg ml^{-1}, respectively.

E. coli BT3Δ*hemG* strain (Δ*hemG*::Kn met_{am3} lac1000 trp_{am} str^r bfe_{am} tsx_{am} su^0) was a gift from Dr. Hachiro Inokuchi (Nagahama Institute of Bio-Science and Technology). This strain was deposited at the National Institute of Genetics with its strain identifier IH85. This strain was grown on Lysogeny Broth (LB) medium (Bertani 1951) supplemented with 2 % glucose in darkness to prevent potential photodamage caused by the accumulation of protoporphyrinogen IX. The *E. coli* competent cells were prepared and transformed according to the method of Inoue et al. (Inoue et al. 1990).

Gloeobacter violaceus PCC7421 (Rippka et al. 1974) was cultured in BG-11 medium (Stanier et al. 1971) using conical flask at 25 ºC on a rotary shaker (NR-80, TAITEC Corp., Saitama, Japan) set at 120 rpm under continuous illumination at 5-10 μmol photons m^{-2} s^{-1} with a fluorescent lamp. The cell growth was traced by measuring $\Delta A_{680-750}$ (corresponding to the amount of chlorophyll *a*) of the cell suspension. Absorption spectra were determined with a laboratory-assembled spectrophotometric system consisting of a high-sensitivity fiber optic spectrometer (QE65Pro, Ocean Optics), an integrating sphere (SPH-6-4, Labsphere), and a halogen light source. To assess the effects of acifluorfen, a pre-culture suspension of *G. violaceus* cells was diluted ($\Delta A_{680-750} = 0.03$) and divided into four flasks. For each flask, either dimethylsulfoxide (DMSO) with or without acifluorfen was added with the final concentration of acifluorfen at 1 mM. Cells were further grown at the same condition to monitor the effects of acifluorfen treatment.

Expression of cyanobacterial PPOX homologues in an *E. coli* Δ*hemG* mutant

The coding sequence of cyanobacterial PPOX homologues, *Gloeobacter hemJ* (gll3040) and *hemY* (glr0943), *Trichodesmium erythraeum hemY* (tlr0374), and *Prochlorocuccus marinus* (P9515_09631), were amplified using genomic DNA isolated from each species as PCR templates. Amplified DNA fragments were cloned into the *Eco*RI site of the plasmid vector pUC119. The plasmid DNA was used for the transformation of the BT3Δ*hemG* strain. The transformants were grown in LB media supplemented with 2 % glucose and 100 μg ml^{-1} ampicillin under darkness with vigorous shaking until the OD_{590} of each culture exceeded 0.1. Every culture was diluted to OD_{590} = 0.1. Each culture was divided into four glass tubes and was inoculated in the same conditions as above for 20 hours.

Transformation of *Synechocystis* with cyanobacterial PPOX homologues

To express PPOX homologues under the control of the *psbA2* promoter in Synechocystis, PPOX homologues were cloned in between the BamHI and XhoI sites of the kmpsbA2 vector (Ito et al. 2008), which contains the *psbA2* promoter, the *nptI* gene of *E. coli,* and the *psbA2* terminator in the backbone of the pGEM-T easy vector. To disrupt the endogenous *hemJ*, we cloned the coding sequence of Synechocystis *hemJ* in pUC119. Then the *E. coli* chloramphenicol acetyl transferase (CAT) gene (Shaw et al. 1979) was inserted into the NheI site of the *hemJ* gene. To replace the *hemJ* gene with PPOX homologues, the *hemJ* gene was cloned together with its upstream (338 bp) and the downstream region (55 bp). The *nptII* gene from *E. coli* was cloned between the stop codon of *hemJ* and its downstream sequence to produce the *hemJΔKn* plasmid. The plasmid DNA was incubated with the Synechocystis cells to allow homologous recombination at the *hemJ* locus which resulted in the knockout of this gene.

Transformation of Synechocystis was carried out by the natural transformation method (Grigorieva and Shestakov 1982) and the transformants were propagated on BG-11 agar plates containing 20 μg/ml kanamycin and/or chloramphenicol. For segregation, transformants were incubated under dim light (approximately 1 μmol photons m^{-2} s^{-1}) with 5 mM glucose. Segregation was checked by PCR every two weeks. If the segregation was not completed, the cells were transferred to new BG-11 agar plates containing higher concentrations of antibiotics, up to 150 μg ml^{-1}.

Measurement of Proto IX

The wild-type (WT) Synechocystis and the *Gv_HemY/ΔhemJ* strains were grown in BG11 until their OD_{750} reached 0.4. Acifluorfen was added to the culture at a final concentration of 0.1 mM. The culture was incubated for three days, and cells were harvested by centrifugation. Porphyrin intermediates and chlorophyll were extracted with dimethylformamide and analyzed by HPLC according to the method of Zapata et al (Zapata et al. 2000) on a Symmetry C8 column (4.6 mm × 150 mm; Waters). During extraction and HPLC analysis, the majority of protoporphyrinogen IX was aerobically oxidized to Proto IX, which emits strong fluorescence. Thus, we detected only Proto IX and other oxidized porphyrins with our fluorescent detector, in addition to chlorophyll, which also produces strong fluorescence. The pigments were monitored by absorbance at 402 nm with a photodiode array detector (L-2450, Hitachi High-Tech Co., Tokyo, Japan) and by fluorescence at 600 nm with a fluorescence detector (L-2485, Hitachi High-Tech), using an excitation wavelength of 417 nm to monitor the majority of chlorophyll intermediates, while Proto IX specifically was monitored at 634 nm by excitation at 400 nm.

Results

Complementation of the *E. coli* Δ*hemG* mutant

In this study, we attempted to validate the functionality of cyanobacterial PPOX homologues by heterologous complementation systems. For this purpose, we selected four PPOX homologues from available genomes: the *hemY* homologue of *Trichodesmium erythraeum* and *G. violaceus*, the *hemG* homologue of *Prochlorococcus marinus* MIT9515, and the *hemJ* homologue of *G. violaceus.* Hereafter, these homologues are called *Te_hemY, Gv_hemY, Pm_hemG* and *Gv_hemJ,* respectively. It should be noted that *G. violaceus* branches early in the phylogeny of cyanobacteria (Rippka et al. 1979; Seo and Yokota 2003) and it contains both *hemJ* and *hemY* homologues. The other cyanobacteria chosen in this study are phylogenetically dispersed in the cyanobacterial phylum (Fig. 1).

All of these genes were amplified by PCR from genomic DNA isolated from respective cultures and cloned into the pUC119 vector under the *lac* promoter. The constructs were then introduced into an *E. coli* strain (BT3Δ*hemG*) in which the *hemG* gene had been disrupted by an insertion of the kanamycin resistance marker. Due to a lack of heme-synthesizing ability, the BT3Δ*hemG* only formed a small colony when grown on a rich medium supplemented with 0.2 %

glucose (Fig. 2a). The *lac* promoter allows a leaky expression of the downstream gene even in media containing a small concentration of glucose in the absence of lactose, which we found was sufficient for our complementation test (Nishimura et al. 1995). Thus, we performed the following complementation test with LB media containing 0.2 % (w/v) glucose, which sustains the viability of BT3Δ*hemG* even when the heme-synthesizing ability was not complemented. As a result, the control strain, to which only the empty pUC119 vector was transformed, and the *Gv_hemJ* expressing strain grew poorly on the media, while the BT3Δ*hemG* strains, which harbored *Gv_hemY, Te_hemY*, or *Pm_hemG*, grew better on the agar media (Fig. 2a), indicating that the latter three genes complemented the *hemG* phenotype of BT3Δ*hemG*.

For further confirmation, we grew these transformants in liquid LB medium containing 0.2 % (w/v) glucose and ampicillin. Similar to the results obtained with plates, the control strain and *Gv_hemJ*-containing strain grew poorly in the liquid medium, while the strains containing *Te_hemY*, *Gv_hemY,* or *Pm_hemG* homologues grew faster and to higher concentrations (Fig. 2b), demonstrating that these three homologues complemented the heme deficient phenotype of BT3Δ*hemG,* while *Gv_hemJ* did not. The maximum cell densities were different for the strains containing either *Te_hemY* and *Gv_hemY,* or *Pm_hemG*, which could be influenced by the depletion of certain elements in the medium, or the accumulation of certain metabolites or aggregated proteins. Nevertheless, as the control strain and the *G. violaceus hemJ*-transformed strain never reached such high densities, we conclude that *T. erythraeum hemY* homologue, *G. violaceus hemY,* and *P. marinus hemG* homologues complemented the heme deficient phenotype of BT3Δ*hemG.*

Replacement of the Synechocystis *hemJ* gene

We examined whether the PPOX homologues were able to substitute the *hemJ* gene of Synechocystis whose function was experimentally verified (Kato et al. 2010). For this purpose, we introduced the same set of PPOX homologues used for *E. coli* transformation into the *psbA2* locus of Synechocystis encoding the D1 subunit of photosystem II. This locus was used because the *psbA2* promoter is relatively strong and is frequently used for the recombination of genes in the research of Synechocystis (Mohamed and Jansson 1989; Wang et al. 2018). As there are three *psbA* loci in the Synechocystis genome, the disruption of the *psbA2* locus does not compromise the growth of the cells (Jansson et al. 1987).

To test whether the PPOX homologues can substitute the function of the *hemJ* gene, we introduced two types of cassettes into the wild type (WT) Synechocystis genome. First, one type of cassette consisting of each PPOX homologue and the kanamycin resistance gene was introduced into the Synechocystis genome to express each PPOX homologue under the control of the *psbA2* promoter (Fig. S1a). After transformants were selected by kanamycin resistance, the other cassette consisting of the chloramphenicol resistance gene which was flanked by the 5' half and the 3' half of the coding region of the endogenous *hemJ* gene (Fig. S1b) were introduced into the Synechocystis genome to disrupt the endogenous *hemJ* locus. All transgenic strains transformed with the two cassettes were grown on BG11 agar plates containing kanamycin (marker for the transformation of PPOX homologues) and chloramphenicol (marker for the disruption of the endogenous *hemJ* gene) and were tested for the disruption of the endogenous *hemJ* locus by PCR. The strains transformed with *Gv_hemY* and *Te_hemY* were homoplasmic in terms of the *hemJ* knock-out (Fig. S1c, d), which indicated that *Gv_hemY* and *Te_hemY* could substitute the *hemJ* gene of Synechocystis (we designated these strains as *psbA-Gv_hemY/ΔhemJ* and *psbA-Te_hemY/ΔhemJ*). On the contrary, the strains transformed with *Gv_hemJ* or *Pm_hemG* remained heteroplasmic containing both the transgenes and the endogenous *hemJ* locus in multiple copies of the genome (data not shown). We speculate that *Gv_hemJ* and *Pm_hemG* could have failed to substitute Synechococcus *hemJ*, because the *psbA2* promoter might be too strong or did not express the PPOX homologues at the required timing.

Subsequently, we used the endogenous *hemJ* promoter to drive the transgene expression, and thus replaced the endogenous *hemJ* gene by directly inserting the gene cassette containing either the *Gv_hemJ* or *Pm_hemG* gene, followed by insertion of the kanamycin resistance marker into the open reading frame of the *hemJ* gene. As a result, *Gv_hemJ* successfully replaced the Synechocystis *hemJ* gene (Fig. S2a), while the cells transformed with *Pm_hemG* remained heteroplasmic (Fig. S2b), indicating that only *Gv_hemJ* complemented the loss of *hemJ* in Synechocystis. We designated these strains as *Gv_hemJ*::*ΔhemJ* and *Pm_hemG*::*ΔhemJ*, though the latter strain was heteroplasmic.

Confirmation of the functionality of HemY-type PPOX by acifluorfen treatment

For the HemY-type PPOX, it is well known that a diphenylether herbicide such as acifluorfen effectively inhibits its activity (Jacobs et al. 1990). To confirm whether *hemY*-transformants (*Gv_hemY/ΔhemJ* and *Te_hemY/ΔhemJ*) are dependent on the transformed *hemY* homologues, we tested their sensitivity to acifluorfen. For this experiment, WT, *psbA2-Gv_hemY/ΔhemJ*, *psbA2-Te_hemY/ΔhemJ*, *Gv_hemJ::hemJ* and *Pm_hemG::hemJ* strains were serially diluted and spotted on BG11 plates containing kanamycin (50 μg ml^{-1}) or acifluorfen (0.1 mM) (Fig. 3). All of these strains were grown on the control BG11 plates which did not contain either kanamycin or acifluorfen (Fig. 3: left). On the BG11 plates containing kanamycin, WT cells did not grow, while the other transformants grew, showing that the transformants contained respective transgenes (Fig. 3: middle). On the BG11 plates containing acifluorfen, the growth of *psbA2-Gv_hemY/ΔhemJ* and *psbA2-Te_hemY/ΔhemJ* were compromised, while the other strains grew and formed colonies (Fig. 3: right). These results demonstrate that replacement of *hemJ* with *hemY* rendered the cells dependent on the HemY-type PPOX. In contrast, WT, *psbA2-Gv_hemJ/ΔhemJ* and *psbA2-Pm_hemG/ΔhemJ* cells were resistant to acifluorfen. The *Te_hemY/ΔhemJ* strain grew slightly on the medium containing acifluorfen. It is possible that the Te_HemY protein is less sensitive to acifluorfen when compared with the Gv_HemY protein. It should be noted that the cells *(Gv_hemJ::hemJ and Pm_hemG::hemJ)* appeared to grow better on the acifluorfen-containing plate (Fig. 3), but we think it could be due to subtle differences in the plate conditions between the agar plates in this particular experiment. In the course of this study, we did not observe any clear indication that acifluorfen had positive or negative effects on the cells except for those containing *hemY* homologues.

Co-existence of HemY and HemJ-type PPOX in *G. violaceus*

The majority of cyanobacteria contain only one of the three PPOX homologues (Kobayashi et al. 2014), implicating that these homologues have redundant functions. On the other hand, *G. violaceus* is an exception in that it contains both *hemY* and *hemJ* homologues in its genome. To investigate if both genes are functional in this species, we treated cells with a high concentration (1 mM) of acifluorfen (Fig. 4). Cells continued to grow regardless of the presence of acifluorfen (Fig. 4). The results imply that *Gloeobacter* is not dependent on the HemY-type PPOX, or that the *Gloeobacter hemY* homologue is not sensitive to this concentration of acifluorfen. Though

we had tested the sensitivity of the Synechocystis *psbA2-Gv_HemY/ΔhemJ* strain to acifluorfen on plates (Fig. 3), we also tested it with a liquid culture of this strain. As a result, the same concentration of acifluorfen significantly suppressed the growth (Fig. S3) and chlorophyll accumulation (Fig. 5) in this strain. Furthermore, we detected ProtoIX in the acifluorfen-treated *psbA2-Gv_HemY/ΔhemJ* strain, indicating that inhibition of Gv_HemY protein compromised tetrapyrrole biosynthesis in the cells (Fig. 5). As an oxidative byproduct of protoporphyrinogen IX, the accumulation of Proto IX suggests that protoporphyrinogen IX accumulated in acifluorfen-treated cells (Jacobs et al. 1991). These results are consistent with those shown in Fig. 3, indicating that the Synechocystis *Gv_HemY/ΔhemJ* strain was dependent on the HemY-type PPOX and that the Gv_HemY protein is sensitive to acifluorfen. A possibility that acifluorfen is not incorporated into *G. violaceus* cells cannot be excluded. However, we think it is unlikely, because acifluorfen is a small compound that can be incorporated into a wide range of algal and plant cells. Thus, we concluded that *G. violaceus* cells are dependent on its HemJ-PPOX under our culture conditions, though they also contain a functional *hemY* gene.

Discussion

In general, phylogenetic analysis can provide a reasonable assumption of whether certain gene homologues encode functional enzymes. According to the phylogenetic analyses of PPOX sequences done by Kobayashi and co-workers (Kobayashi et al. 2014), the *hemG* homologues of cyanobacteria form a clade with the *hemG* genes of Gammaproteobacteria, among which the functionality of the *hemG*-encoded enzyme of *E. coli* was verified by an *in-vitro* enzyme assay (Boynton et al. 2011). This analysis indicates that the cyanobacterial *hemG* homologues may encode functional HemG-type PPOX enzymes. Likewise, the majority of cyanobacterial *hemJ* homologues form a single clade that includes the Synechocystis *hemJ* gene that has been shown to encode a functional subunit of the enzyme (Boynton et al. 2011; Kato et al. 2010; Skotnicová et al. 2018). Cyanobacterial *hemY* homologues also form a small clade that are a part of a bigger clade that includes plant *hemY* genes, which are shown to encode functional enzymes (Kobayashi et al. 2014). Nevertheless, there are sometimes exceptions for the assumption on functional homology based on phylogenetic analysis. For example, a clade for chlorophyll *b* reductase contains a group of sequences from organisms that do not contain chlorophyll *b* (Lim et al.

2019), thus, these sequences should not be considered chlorophyll *b* reductases. In another case, a clade for ferredoxin-dependent divinyl-chlorophyllide reductase contains those for 7-hydroxymethyl chlorophyll *a* reductase (Meguro et al. 2011). Thus, we provided experimental evidence for the functionality of all three types of PPOX enzymes (HemG, HemY and Hem J) co-distributed throughout the cyanobacterial phylum and showed that two types of PPOX enzymes (HemY and HemJ) exceptionally coexist in one cyanobacterial species *G. violaceus* by analyzing the functionality of the *hemY* and *hemJ* homologues in this study.

In addition, we had a second objective of our study, which was to analyze whether the three PPOX genes are functionally interchangeable in order to obtain an insight into potential horizontal transfer events of these genes among cyanobacterial strains. It is hypothesized that *hemY* is the most ancestral form of cyanobacterial PPOX (Kobayashi et al. 2014), because *hemY* is most widely distributed among bacteria (Kato et al. 2010; Kobayashi et al. 2014), and because *hemY* also exists in *G. violaceus,* which is considered one of the earliest branching primordial cyanobacterial species (Honda et al. 1999). In our observation, the HemY-type PPOX is more versatile than the other two types of PPOX at least under our experimental conditions, as it could replace the HemJ-type and HemG-type PPOX in both cyanobacteria and Gammaproteobacteria, respectively (Figs. 2, 3). Accordingly, we speculate that horizontal transfer of *hemY* to other bacteria could be relatively easy and could lead to rapid distribution of this gene among bacteria. We also speculate that an increase in the atmospheric oxygen concentrations by ancient cyanobacteria might have enhanced the distribution of HemY-type PPOX, which utilizes oxygen as a terminal electron acceptor (Dailey et al. 1994). According to Kobayashi et al. (Kobayashi et al. 2014) the phylogenetic trees of both *hemY* and *hemJ* mostly match that of cyanobacterial 16S rRNA, indicating that the *hemY* and *hemJ* genes were present in ancestral cyanobacteria and were vertically transferred to the descendant cyanobacteria. In their analysis, *hemJ* of *G. violaceus* clustered with that of the *Anabaena* or *Synechococcus-Prochlorococcus* lineages, which indicates that the *hemJ* gene of *G. violaceus* was obtained by horizontal gene transfer. However, the bootstrap value for this clustering was low, and we think the hypothesis that *hemJ* is more ancestral is also possible.

In contrast to *hemY* or *hemJ,* the distribution of *hemG* is limited to two *Prochlorococcus* species in the cyanobacterial phylum. Since these two *hemG* genes form a cluster with the *hemG*

genes of Gammaproteobacteria (Kobayashi et al. 2014), it is most likely that they were transferred from a Gammaproteobacteria to the *Prochlorococcus* species. Interestingly, the *hemG* genes in these cyanobacteria are located in the same region of the genomes as *hemJ* in the other *Prochlorococcus* species (Kobayashi et al. 2014), which is another line of evidence that shows *hemJ* was replaced by *hemG* in these species. Nevertheless, the *Prochlorococcus hemG* did not completely replace the *hemJ* locus of Synechocystis (Fig. S2b). There may be unknown conditions or factors that are necessary to utilize HemG-PPOX in cyanobacteria. Boynton et al. (2009) showed that HemG-type PPOX requires menadione as an electron acceptor. Menadione is an intermediate of the biosynthesis of menaquinone or phylloquinone, which is an essential component of cyanobacterial photosystem I. Thus, it is likely that menadione exists as an intermediate of menaquinone biosynthesis in cyanobacteria. We speculate that the menadione levels may vary among cyanobacteria, which could coincide with the ease of *hemG* complementation. On the other hand, the level of menadione must not be a limiting factor for complementation in *E. coli,* which explains why *Pm_hemG* complemented the BT3Δ*hemG* strain.

In *G. violaceus,* we showed that the two PPOX homologues are both functional. On the contrary, the majority of cyanobacteria contain only one type of PPOX, which indicates that one type of PPOX is sufficient for the synthesis of Proto IX. It is possible that *G. violaceus* can switch between its two PPOX enzymes depending on environmental or growth conditions. This cyanobacterium is a common rock-dwelling organism (Mareš et al. 2013), which may experience drastically changing light intensities and humidity. It is likely that the HemY-type and HemJ-type PPOX may have distinct selective advantages under different environmental conditions. For example, under oxidative conditions, HemY-PPOX may have better activity compared with HemJ-PPOX. In our liquid culture conditions, where light intensity is relatively low, acifluorfen treatment did not affect the growth of *G. violaceus,* which indicates that HemJ-PPOX is active enough to compensate the inhibition of HemY-PPOX under such conditions. A better understanding of the reaction mechanism of HemJ-PPOX is needed to assess the advantage of each type of PPOX under different conditions.

The difference in the two complementation systems demonstrate their limitation in verifying the functionality of certain genes. There are several possible reasons that could lead to

failure of complementation. It was possible that a certain substance or protein was insufficient or missing in the host cell. For example, menaquinone is required for the activity of the HemG-type PPOX (Boynton et al. 2009). Though it was likely that Synechocystis cells are able to synthesize menaquinone as an intermediate of phylloquinone synthesis, it was possible that the cellular level of menaquinone was not sufficient for the activity of the HemG-type PPOX. Otherwise, if a PPOX enzyme needs an interaction with another protein, this interaction could be insufficient in a host cell. As it is mentioned in the Introduction section, the HemJ-type PPOX may need an association with coproporphyrinogen III oxidase, this interaction could be too weak for the activity of the HemJ-type PPOX in *E. coli.* It was also possible that the expression of the transformed enzyme was not expressed in a sufficient quantity to fulfil the cellular demand of the cells. When we first introduced *Te_hemY* and *Gv_hemY* into Synechocystis under the control of the *psbA2* promoter, these genes did not complement *hemJ* deficiency. In contrast, introducing *Te_hemY* and *Gv_hemY* into the *hemJ* locus of Synechocystis was successful (Fig. S1). We speculate that cellular requirement for tetrapyrroles including heme, chlorophyll, and bilins may be different from that of D1 protein encoded by *psbA2.* D1 is only necessary under illumination, while certain tetrapyrrole species, such as heme, are required both under illumination and darkness. In addition, it would be reasonable to assume that the expression level of the PPOX gene is coordinated with those of other tetrapyrrole biosynthesis genes in cells.

Though there is a limitation in a single complementation system to verify a functionality of an enzyme as discussed above, other experimental systems may also have limitations. *In vitro* assay of enzyme activities could be a reliable option, but in the case of HemJ-type PPOX, detection of *in vitro* activity requires an experimental setup under anoxygenic conditions, and it could fail for some species without specific reasons. Thus, a combination of two heterologous complementation systems, as it is shown in this study, represents a simple and practical way to verify the function of an enzyme.

In conclusion, our complementation experiments show the functionality of three types of PPOX homologues distributed in the cyanobacterial phylum in a mosaic pattern, indicating that their function agrees with sequence similarity and phylogenetic analysis (Kato et al. 2010; Kobayashi et al. 2014), which may provide clues to the evolution of PPOX enzymes in the

bacterial domain. Our study also showed the feasibility and limitation of the two heterologous complementation systems for PPOX enzymes.

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Fig. 1 Distribution of PPOX homologues in the cyanobacterium phylum is drawn on a dendrogram showing phylogenetic relationships of representative cyanobacteria. The phylogeny was deduced from the average sequence similarities of the genome (Satoh et al. 2013). The node lengths do not reflect the distance between each pair of organisms. The strains from which we tested the functionality of PPOX homologues are indicated by the red frames. *Synechocystis* sp. PCC6803, which we used for transformation in this study, is indicated by a star. To the right side of the tree, the results of the complementation analysis are shown with "+" and "-" indicating success and failure of complementation, respectively.

Fig. 2 Complementation of the BT3Δ*hemG* strain of *E. coli* with cyanobacterial PPOX homologues. GvHemY, TeHemY, GvHemJ, PmHemG denote the transformants with *Gloeobacter violaceus hemY* homologue, *G. violaceus hemJ* homologue, *Trichodesmium erythraeum hemY* homologue and *Prochlorococcus marinus hemG* homologue, respectively. Control is the BT3Δ*hemG* strain transformed with the pUC119 vector. (**a**) Growth of the BT3Δ*hemG* transformants on an Lysogeny Broth (LB) agar plate with 0.2 % (w/v) glucose supplemented. Single colonies of each transformant were streaked on a plate and incubated overnight at 37 °C. (**b**) Growth of the BT3Δ*hemG* transformants on LB liquid media containing 0.2 % (w/v) glucose in darkness. Single colonies of each transformant were precultured overnight at 37 °C and diluted to the same optical density at 590 nm for each strain. Three cultures were prepared for each strain from a single preculture.

Fig. 3 Complementation of Synechocystis *HemJ* by PPOX homologues of other cyanobacteria. Preculture of wild type (WT) Synechocystis, and *psbA2-Gv_hemY/ΔhemJ*, *Gv_hemJ::hemJ, psbA2-Te_hemY/ΔhemJ,* and *Pm_hemG::hemJ* strains were diluted and spotted with serially diluted cell numbers as follows: 1: 3×10^5 cells, 2: 3×10^4 cells, 3: 3×10^3 cells, and 4: 3×10^2 cells. The cells were incubated for two days at 21 °C under 30 μmol photons m^{-2} s^{-1}. Left: BG-11 without kanamycin (Kn) or acifluorfen (Acf). Middle panel: BG11 with 50 μg ml^{-1} (w/v) of kanamycin. Right: BG-11 with acifluorfen. We marked the *Pm_hemG::hemJ* strain with an asterisk to indicate that this strain was heteroplasmic containing the endogenous *hemJ* genes in the multi-copy genome (see Fig. S2).

Fig. 4 Effect of acifluorfen on the growth of *Gloeobacter violaceus.* A culture suspension of *G. violaceus* was divided into four batch when $\Delta A_{680\text{-}750}$ was ~0.06 (after 140 h preculture) where indicated by an arrow in the graph. For one flask, DMSO was added at the final concentration of 0.1 % (v/v), which is shown with a dashed line with crosses. For the other two flasks, acifluorfen was added at the final concentration of 1 mM, which is shown with triangles and squares, respectively. For control cells (open circles), no additional compounds were supplemented. Cells were further grown for 143 hours to monitor the effects of acifluorfen treatment.

Fig. 5 Proto IX accumulation in the *psbA2-Gv_hemY/ΔhemJ* strain upon treatment of acifluorfen. When the WT Synechocystis and the *psbA2-Gv_hemY/ΔhemJ* strain were grown in BG-11 until OD_{750} reached 0.4, acifluorfen was added to the culture at a final concentration of 0.1 mM. The cells were cultured for an additional three days as shown in the photographs next to the chromatograms. Then, pigments were extracted with dimethylformamide and analyzed by HPLC as described in Materials and Methods. Representative HPLC chromatograms monitored by absorbance at 402 nm for WT (**a**) and *psbA2-Gv_hemY/ΔhemJ* (**b**) are shown. Insets show the HPLC chromatograms monitored by a fluorescence detector with which the excitation/detection wavelengths were set for the detection of chlorophyll *a* and other Mg-containing tetrapyrroles (excitation at 417 nm and detection at 600 nm) from 0 to 10 min and 15 to 27 min, while the combination of the excitation/detection wavelength were switched for Proto IX detection (excitation at 400 nm and detection at 634 nm) between 10 to 15 min as indicated by blue bars on the X axis.

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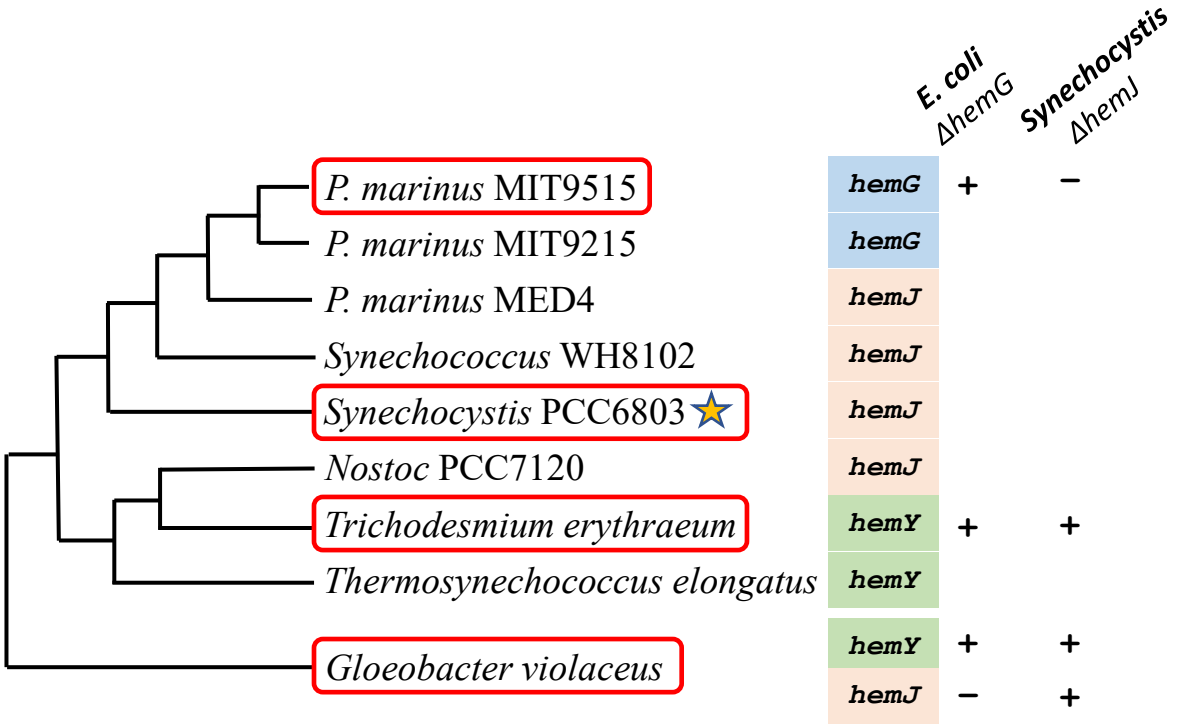
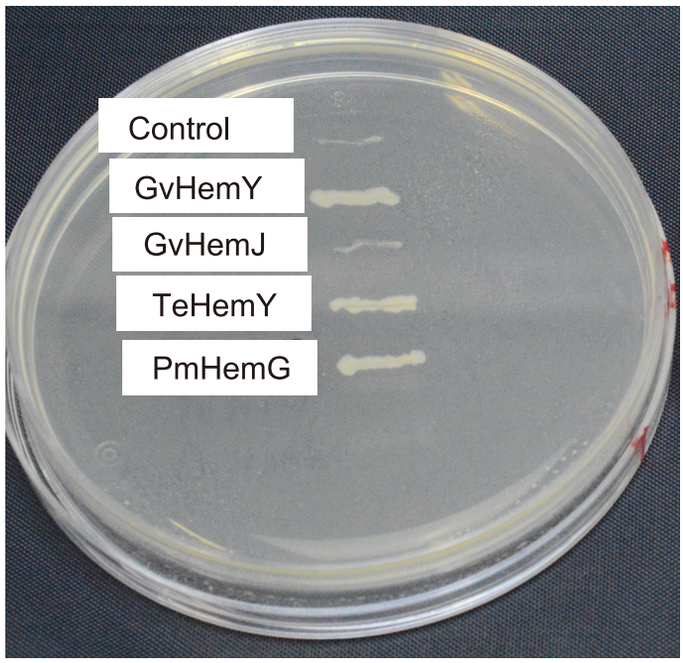
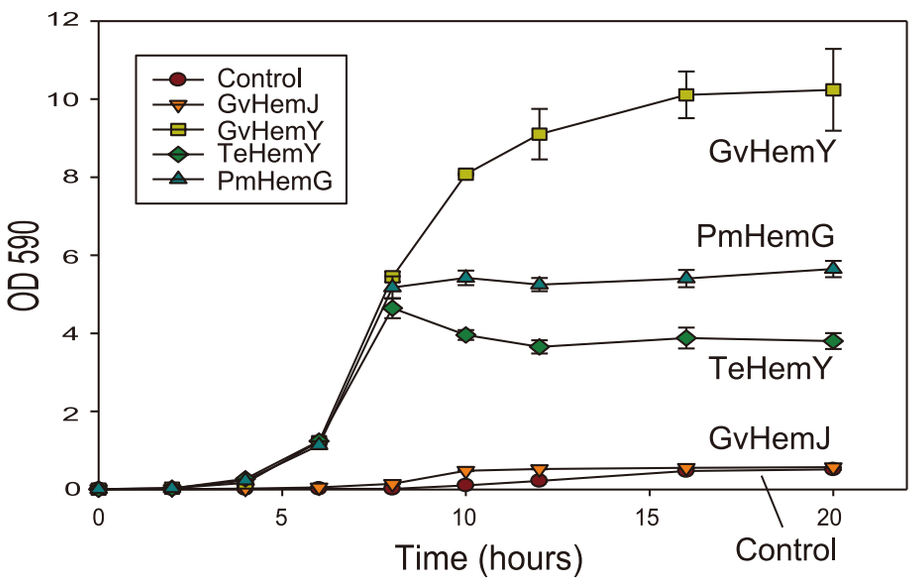

E. coli ΔhemG
Synechocystis ΔhemJ
P. marinus MIT9515
P. marinus MIT9215
P. marinus MED4
Synechococcus WH8102
Synechocystis PCC6803
Nostoc PCC7120
Trichodesmium erythraeum
Thermosynechococcus elongatus
Gloeobacter violaceus
hemG + −
hemG
hemJ
hemJ
hemJ
hemJ
hemY + +
hemY
hemY + +
hemJ − +

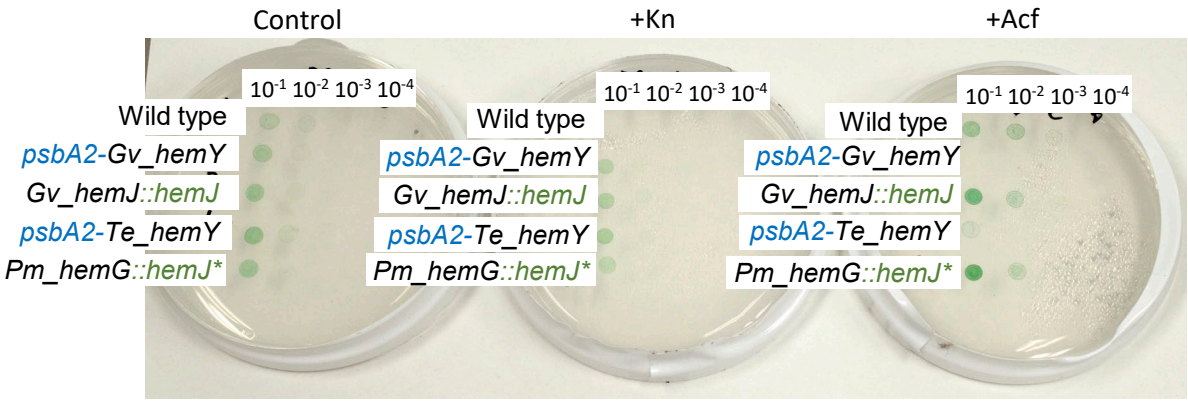
Figure 1

a


Control
GvHemY
GvHemJ
TeHemY
PmHemG

b


Control
GvHemJ
GvHemY
TeHemY
PmHemG
OD 590
Time (hours)
GvHemY
PmHemG
TeHemY
GvHemJ
Control



**Pm_hemG::hemJ* is heteroplasmic.

Figure 3

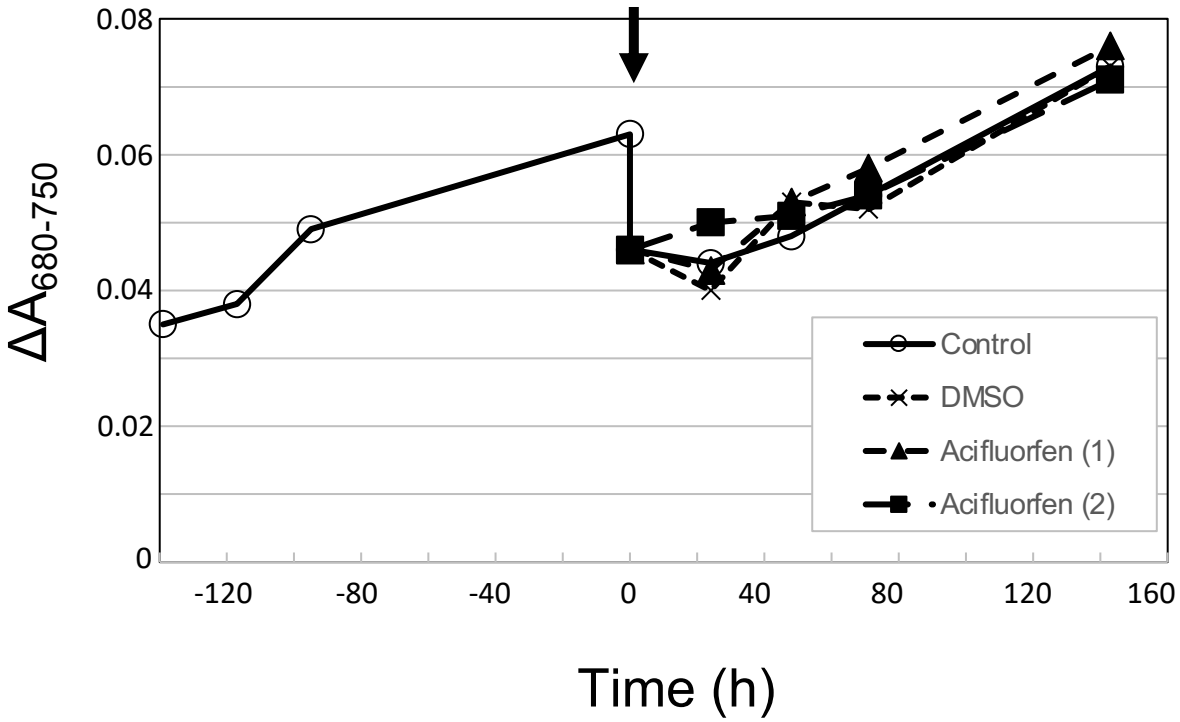

0.08
0.06
0.04
0.02
0
ΔA680-750
-120
-80
-40
0
40
80
120
160
Time (h)
Control
DMSO
Acifluorfen (1)
Acifluorfen (2)

Figure 4

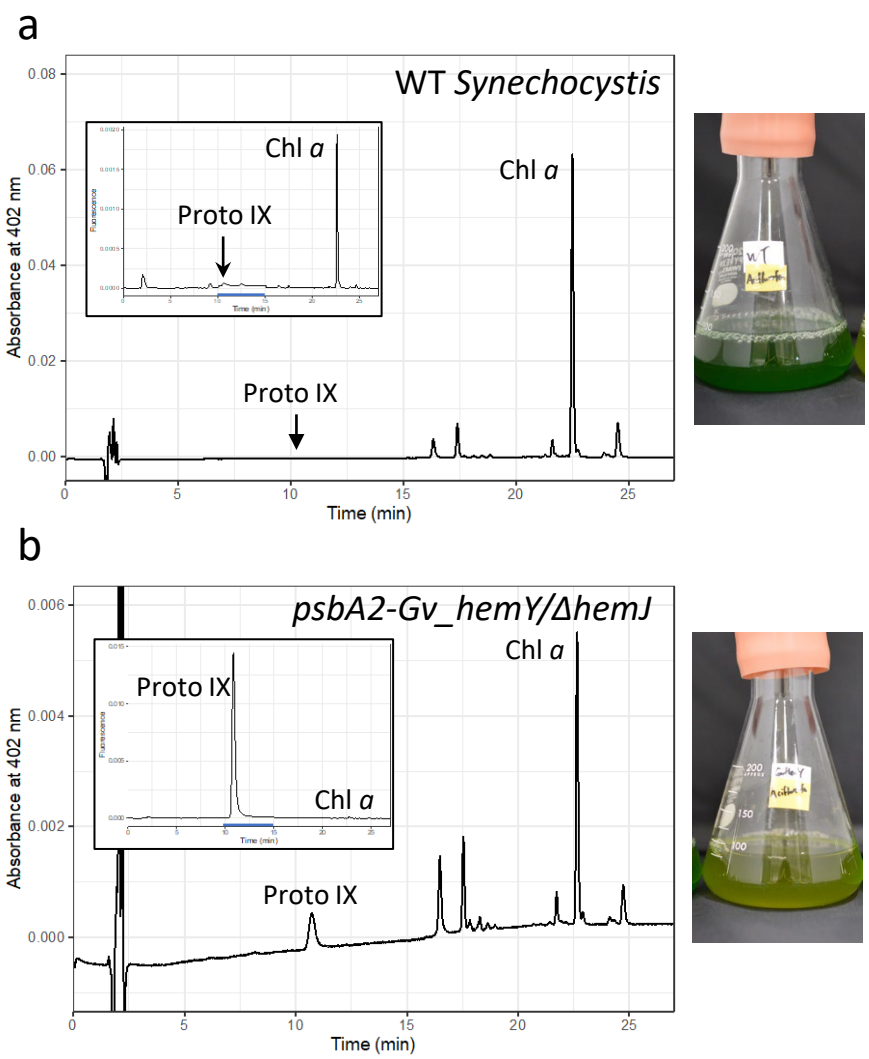

a
WT Synechocystis
Chl a
Proto IX
Chl a
Proto IX
Absorbance at 402 nm
Time (min)
b
psbA2-Gv_hemY/ΔhemJ
Chl a
Proto IX
Chl a
Proto IX
Absorbance at 402 nm
Time (min)

Figure 5