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Characterization of the genuine type 2 chromatic acclimation in the two *Geminocystis* cyanobacteria

Yuu Hirose[1,*], Naomi Misawa[1], Chinatsu Yonekawa[1], Nobuyoshi Nagao[1], Mai Watanabe[2], Masahiko Ikeuchi[2], and Toshihiko Eki[1]

[1]Department of Environmental and Life Sciences, Toyohashi University of Technology, 1-1 Hibarigaoka, Tempaku, Toyohashi, Aichi 441-8580, Japan, and [2]Department of Life Sciences (Biology), The University of Tokyo, 3-8-1 Komaba, Meguro, Tokyo 153-8902, Japan

*To whom correspondence should be addressed. Tel. 81-532-44-6912. Fax. 81-532-44-6929. Email: hirose@ens.tut.ac.jp

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Abstract

Certain cyanobacteria can adjust the wavelengths of light they absorb by remodeling their photosynthetic antenna complex phycobilisome via a process called chromatic acclimation (CA). Although several types of CA have been reported, the diversity of the molecular mechanisms of CA among the cyanobacteria phylum is not fully understood. Here, we characterized the molecular process of CA of *Geminocystis* sp. strains National Institute of Environmental Studies (NIES)-3708 and NIES-3709. Absorption and fluorescence spectroscopy revealed that both strains dramatically alter their phycoerythrin content in response to green and red light. Whole-genome comparison revealed that the two strains share the typical phycobilisome structure consisting of a central core and peripheral rods, but they differ in the number of rod linkers of phycoerythrin and thus have differing capacity for phycoerythrin accumulation. RNA sequencing analysis suggested that the length of phycoerythrin rods in each phycobilisome is strictly regulated by the green light and red light-sensing CcaS/R system, whereas the total number of phycobilisomes is governed by the excitation-balancing system between phycobilisomes and photosystems. We reclassify the conventional CA types based on the genome information and designate CA of the two strains as genuine type 2, where components of phycoerythrin, but not rod-membrane linker of phycocyanin, are regulated by the CcaS/R system.

Key words: Cyanobacteria, chromatic acclimation, phycobilisome, cyanobacteriochrome

1. Introduction

Photosynthetic organisms have evolved various photoacclimation mechanisms to perform photosynthesis under varying light conditions. Cyanobacteria are Gram-negative prokaryotes that perform oxygenic photosynthesis using two photosynthetic reaction centers, namely photosystems II and I.[1] They utilize an antenna complex called the phycobilisome, which harvests and transfers light energy to the photosystems.[2,3] The typical phycobilisome comprises a central core and multiple peripheral rods, each of which consists of tetrapyrrole-bound phycobiliproteins and non-chromophorylated linker proteins.[2,3] Most cyanobacteria utilize the phycobiliproteins allophycocyanin (APC) and phycocyanin (PC) for the core and rods,

respectively. Certain species utilize the additional phycobiliprotein phycoerythrocyanin or phycoerythrin (PE) for the rods. These phycobiliproteins absorb light of differing wavelengths: 650–670 nm for APC, 620–630 nm for PC, 550–620 nm for phycoerythrocyanin, and 490–570 nm for PE. These phycobiliproteins are organized by linker proteins to allow directional energy transfer from distal phycoerythrocyanin or PE to proximal PC within the rod, then to APC in the core, and finally to the photosystems. Interestingly, direct interaction of the PC rods to the photosystems has been reported in some strains,[4,5] suggesting the diversity of light harvesting processes in the cyanobacteria phylum.

Certain cyanobacteria maximize their light-harvesting capacity by remodeling phycobilisome components, which is called chromatic acclimation (CA). In the 1970s, cyanobacterial species that produce both PE and PC were classified into three groups based on changes in their PE and PC content in response to green and red light.[6] Group I species show no change in PC or PE content in response to green or red light. Group II species accumulate PE under green light but not under red light, and they constitutively produce PC. Group III species accumulate PE under green light but not under red light, whereas they accumulate PC under red light but not under green light. The CA of Group II and Group III species are called CA of 'type' 2 (CA2) and 'type' 3 (CA3), respectively,[7] and there is no CA1. CA3 is also referred to as complementary CA because cell color changes complementary to the ambient light color.[7,8] CA2 and CA3 occur in terrestrial and freshwater cyanobacteria.[6] In the 2000s, marine cyanobacteria were shown to change the composition of their tetrapyrrole chromophores of PE, blue light-absorbing phycourobilin, and green light-absorbing phycoerythrobilin. This is called CA of type 4 (CA4) and occurs in marine *Synechococcus* lineages.[9,10] Recently, certain terrestrial cyanobacteria have been shown to remodel proteins of the phycobilisome core and reaction center of photosystems with synthesis of chlorophyll *f* or *d* under far-red light.[11] This acclimation is called far-red light photoacclimation, and it is critical for species deep within bacterial mats, where only far-red light is available.

Photosensors for these photoacclimation processes of cyanobacteria have been identified by forward and reverse genetic approaches.[7,8,12] CA3 and CA2 are regulated by the cyanobacteriochrome-class photosensors RcaE and CcaS,[13,14] respectively, which utilize the change of bilin protonation state for their unique green/red absorption change.[15] In CA3, RcaE phosphorylates response regulator RcaC via phosphotransfer protein RcaF under red light.[13,16,17] Phosphorylated RcaC binds to the inverted sequence motif called the L-box in the promoter of the genes encoding PC and phycocyanobilin synthase and activates their expression.[17] Phosphorylated RcaC also binds to the promoter of PE genes to repress their expression. In CA2, CcaS phosphorylates response regulator CcaR under green light.[14,18] The phosphorylated CcaR binds to the direct repeat sequence called the G-box in the promoter of PE genes, and activates their expression.[18] FaRLiP is regulated by RfpA, which is a canonical phytochrome of the knot-less type and undergoes a red/far-red photocycle.[11] RfpA phosphorylates response regulator RfpB via phosphotransfer protein RfpC under far-red light[19]. Phosphorylated RfpB induces far-red–adapted subunits of APC, photosystem II, photosystem I.[11] The photosensor for CA4 has not been identified, but CA4 is regulated at the transcriptional level by the two response regulators FciA and FciB.[20] The RcaE/F/C, CcaS/R and RfpA/C/B systems are distributed in many, but not all, cyanobacteria genomes with weak phylogenetic relationships,[12,21] suggesting that horizontal gene transfer played an important role in the evolution of these photoacclimation systems.

To explore variations in the CA process, we focused on CA-capable cyanobacteria *Geminocystis* sp. strains National Institute of Environmental Studies (NIES)-3708 and NIES-3709, whose complete genome sequences were previously determined by our group.[22,23] We performed spectroscopic analysis and genome and transcriptome comparisons of strains NIES-3708 and NIES-3709 in response to green and red light. We demonstrate that the two strains share the common process of CA regulated by the CcaS/R system but that differences in the structure of PE rods underlie the observed differences in the cellular PE content between the two strains. In addition, we propose to reclassify CA based on the gene structure of the CcaS/R cluster to include cyanobacteria that produce PC but not PE. Our study expands our understanding of the diversity of CA processes in the cyanobacteria phylum.

2. Materials and methods

2.1. Strains, growth conditions, and spectral analyses

Geminocystis sp. strains NIES-3708 and NIES-3709 were obtained from the culture collection of the NIES of Japan. Cells were grown in liquid BG11 medium continuously bubbled with air containing 1% (v/v) CO_2 at 30 °C. Cells were irradiated with 20 μE m^{-2} s^{-1} green light (peak at 524 nm) or 15 μE m^{-2} s^{-1} red light (peak at 658 nm), which was supplied by colored fluorescent lamps (FL20S-G or FL20S-R; Panasonic). Emission lines of the fluorescent lamps were removed by a green or red filter (Supplementary Fig. S1). Cellular absorption spectra were obtained with a UV-Vis spectrophotometer (Model V-650; JASCO) equipped with a transmission integrating sphere (Model ISV-722; JASCO). Low-temperature fluorescence emission and excitation spectra at 77K were obtained with a fluorescence spectrometer (Model FP8300; JASCO) equipped with a liquid nitrogen cooling unit (Model PMU-830; JASCO).

2.2. Isolation of phycobilisome

Cells were harvested by centrifugation, and washed with 0.65 M potassium phosphate buffer (pH 8.0). Cells volumes were adjusted to 100 μl, resuspended in 1 ml of the phosphate buffer, and broken using a TyssueLyser II (Qiagen) with 0.9 g of zirconia/silica beads (0.1 mm diameter) at 30Hz for 1 min at room temperature. The cell extract was treated with 1.8% (vol/vol) Triton X-100 in the phosphate buffer for 30 min in the dark and centrifuged at 20,000 × *g* for 20 min at 18 °C. The supernatant was loaded onto a 10–50% (wt/vol) linear sucrose density gradient in the phosphate buffer and then centrifuged at 130,000 × *g* for 12 h at 18 °C.

2.3. Genome comparison

Nucleotide sequences of accessions AP014815 through AP014820 for strain NIES-3708 and AP014821 through AP014832 for strain NIES-3709 were downloaded from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/ (1 March 2017, date last accessed)). The genome synteny plot of the chromosomes of the two strains was drawn using GenomeMatcher.[24] Circular plots of the chromosomes and plasmids were drawn using ArcWithColor ver. 1.4, an accessory tool of GenomeMatcher. Protein alignments were analysed using Clustal X.[25] Homology searches were preformed using BLAST+.[26] The phylogenetic tree was prepared using FigTree (http://tree.bio.ed.ac.uk/software/figtree/ (1 March 2017, date last accessed)).

2.4. RNA sequencing analysis

Total RNA was extracted from 70-ml *Geminocystis* liquid cultures at mid-logarithmic growth phase. Liquid cultures were cooled quickly with ice and then centrifuged at 4000 × *g* for 5 min at 4 °C. Cell pellets were transferred to microtubes and centrifuged again; then 400 µl buffer RLT of the RNeasy Mini kit (Qiagen) containing 1% (v/v) β-mercaptoethanol and 0.5 g zirconia/silica beads was added to each pellet. Cells were broken for 1.5 min by TissueLyser II at 30 Hz. The extracted RNA was purified using the RNeasy Mini kit. Genomic DNA was digested using the RNase-free DNase set (Qiagen). Ribosomal RNA was removed using the Ribo-Zero rRNA Removal kit for Bacteria (Illumina). RNA libraries were prepared using the TruSeq Stranded mRNA Library Preparation kit (Illumina). Single end of the libraries were sequenced on the MiSeq instrument with the MiSeq Reagent kit v2 (50- cycles; Illumina). Read mapping was performed using BWA version 0.7.12[27] (Supplementary Tables S3 and S4). The mapped reads were counted using HTSeq version 0.6.1,[28] and the counts were normalized as reads per kilobase of CDS per million mapped reads (RPKM).

3. Results and discussion

3.1. Spectroscopic analyses of CA

During the cultivation of the cells of *Geminocystis* sp. strains NIES-3708 and NIES-3709, we noticed that cellular colors of the two strains are different, though they belong to the same *Geminocystis* genus. Therefore, we cultured cells of the two strains under continuous green or red light and characterized their properties of CA. Photomicrographs of the cells showed that the NIES-3708 cells were spherical, with ∼5-µm diameter (Fig. 1A). The NIES-3709 cells were slightly oval, with ∼5 and ∼3 µm longer and shorter axes, respectively, and they contained granular structures (Fig. 1A). Under green light NIES-3708 cells appeared brown, whereas NIES-3709 cells were reddish brown (Fig. 1A). Under red light, both strains appeared blue-green (Fig. 1A). Cellular absorption spectra, which were normalized to chlorophyll at 677 nm, showed that both strains accumulated PE under green light (peak, 567 nm) but not under red light, whereas they accumulated PC under both green and red light (peak, 623 nm; Fig. 1B and C). The peak wavelength of chlorophyll, PE, and PC were the same between the two strains and both light conditions.

Phycobilisomes of the two strains, which were fully acclimated to green or red light, were isolated with sucrose density gradient, yielding two major fractions. The lower and upper fractions were designated to fraction 1 and fraction 2, respectively (Fig. 1D). Absorption spectra showed that the fraction 1 contains PE (peak, 566 nm), PC (peak, 618-620 nm), and APC (shoulder, 650 nm) (Fig. 1E). On the other hand, the fraction 2 contains small amount of chlorophylls (peak, 676 nm) and no detectable APC (Fig. 1F). Low-temperature fluorescence emission spectra showed that the fraction 1 has maximum emission from APC (peak, 683–684 nm), whereas the fraction 2 has maximum emission from PC (peak, 649–650 nm) (Fig. 1G and H). Low-temperature fluorescence excitation spectra of the fraction 1 are similar to absorption spectra of that (Fig. 1I and E), suggesting that both PE and PC efficiently transfer light energy to APC (Fig. 1F). These results indicate that the fraction 1 contains the intact phycobilisome, whereas the fraction 2 contains dissociated rods consisting of PE and PC. Notably, the positions of the fraction 1 are lower under green light than under red light in the two strains (Fig. 1D). In addition, heights of absorption peaks of PC relative to APC in the fraction 1 do not change in the two strains (Fig. 1E). These results indicate that both strains do not replace PC rods chromatically, but they synthesize additional PE rods in the peripheral position of PC rods under green light, which is classified as CA2 by the conventional classification of CA.[6]

The two strains were originally isolated from freshwater environment where other cyanobacteria or algae compete for light energy. Notably, strain NIES-3709 accumulated more PE than NIES-3708 under green light (Fig. 1E and F). On the other hand, strain NIES-3708 repressed synthesis of PE more completely than NIES-3709 under red light (Fig. 1E and F). These findings suggest that strain NIES-3709 has been adapted to green light rich environment more than strain NIES-3708 does. During cultivation of the cells in the liquid medium with bubbling, the two strains form cellular aggregates, and strain NIES-3709 shows more severe aggregation than strain NIES-3708 does (data not shown). Self-shading due to aggregation or competition for light energy with other organisms due to sedimentation of aggregates might produce the different capacity of PE accumulation between the two strains.

3.2. Whole-genome comparison

To explore the molecular mechanisms underlying the different CA responses, we compared the gapless complete genome sequences of strains NIES-3708 and NIES-3709, which were previously determined by our group.[22,23] Sequence identity of the 16S rRNA gene between the two strains was 97.5%. Genome synteny of the chromosome was not conserved between the two strains (Supplementary Fig. S2). These findings suggest that the two strains are distantly related to each other although they both belong to genus *Geminocystis*. Therefore, we compared the gene content of the two strains at the amino acid level (Supplementary Tables S1 and S2). First, orthologous genes were predicted based on bidirectional best hits in the BLASTP program with e-value <E-5 (Fig. 2A).[26] Second, the paralogous genes were predicted based on unidirectional best hits in BLASTP using translated protein sequences that were not assigned to orthologs as the query and the orthologous protein sequences as the subject. Finally, genes that were not assigned as orthologs or paralogs were considered unique genes. A total of 2,714 genes were predicted to be orthologs in the two strains (Fig. 2B; Supplementary Fig. S3). For each of NIES-3708 and NIES-3709, 342 and 385 genes were predicted to be paralogs and 583 and 838 genes were predicted to be unique, respectively (Fig. 2B; Supplementary Fig. S3). We compared the coverage, identity, and e-values from BLASTP of the orthologs and paralogs. Most of the orthologs had high coverage, high identity, and low e-values (Fig. 2C), suggesting that ortholog prediction based on bidirectional best hit is a simple but reliable approach. On the other hand, coverage and identity of the paralogs rapidly decreased with increasing e-value (Fig. 2D), suggesting that the predicted paralog set may contain some unique genes. Conversely, this means that the confidence was high in our prediction of the unique genes.

Genes encoding the phycobilisome components and its associated proteins are highly conserved in strains both NIES-3708 and NIES-3709 although those of strain NIES-3709 are more scattered across the genome compared with NIES-3708 (Fig. 3A). For biosynthesis of the APC core, both NIES-3708 and NIES-3709 harbor two copies of genes for the core subunit (*apcB1A1, apcB2, acpA2*), small core linker (*apcC*), and chromophorylated large core membrane linker (*apcE*). For biosynthesis of the PC rod, both strains harbor single-copy genes for the core subunit (*cpcBA*), rod-core linker (*cpcG*), phycocyanobilin chromophore synthase (*pcyA*), and chromophore lyases

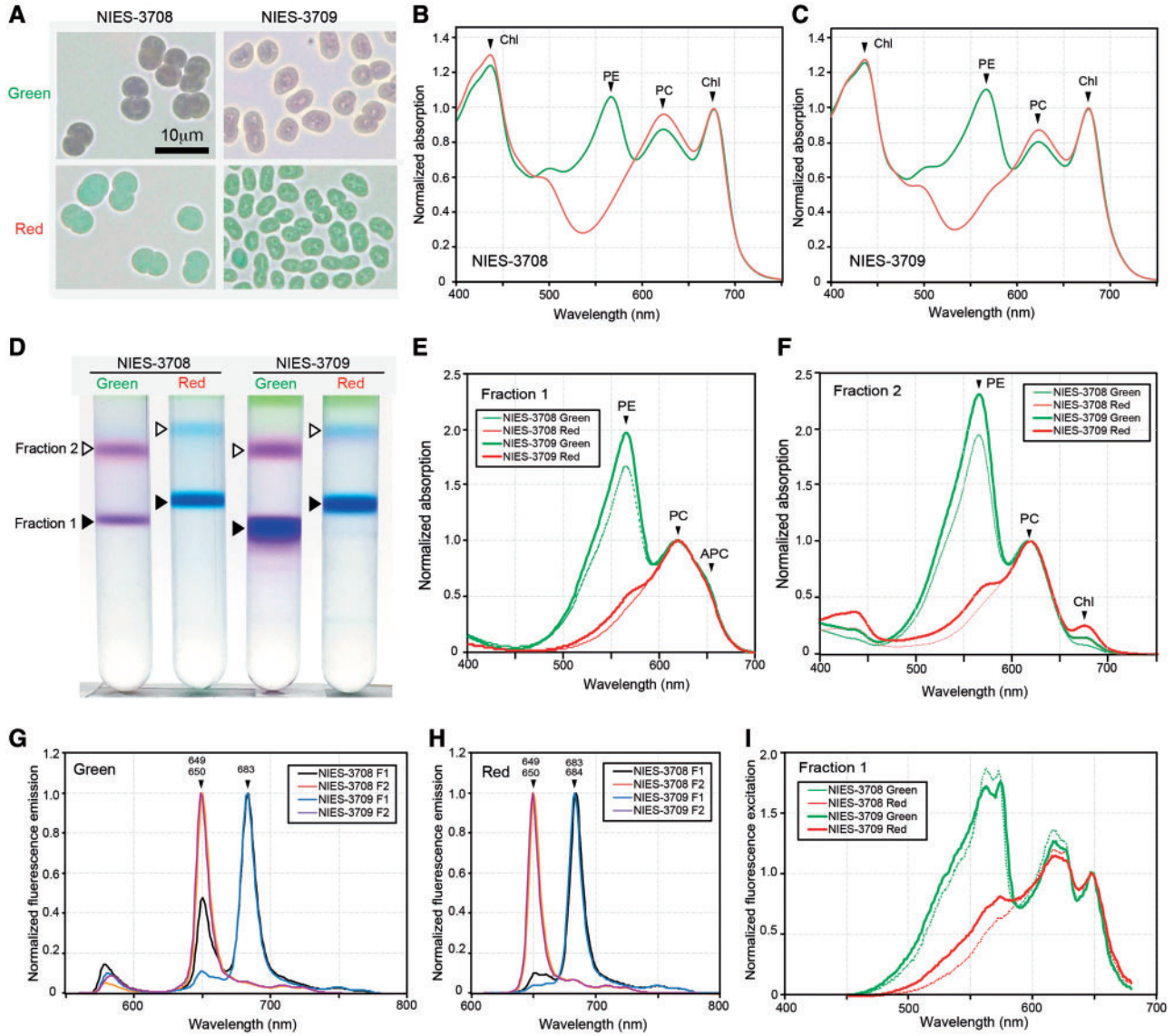

Figure 1. Characterization of CA of two *Geminocystis* strains. A, Micrographs of *Geminocystis* sp. NIES-3708 and NIES-3709 grown under green light or under red light. B and C, Cellular absorption spectra of strains NIES-3708 (B) and NIES-3709 (C) grown under green light (green line) or under red light (red line). Spectra were normalized at chlorophyll peaks. D, Isolation of phycobilisome from strains NIES-3708 and NIES-3709 grown under green or red light. Fraction 1 and fraction 2 were indicated by closed and open triangles, respectively. E and F, Absorption spectra of fraction 1 (E) and fraction 2 (F) isolated form strains NIES-3708 (thin dashed line) and NIES-3709 (thick solid line) grown under green light (green line) or under red light (red line). Spectra were normalized at PC peaks. G and H, 77K low-temperature fluorescence emission spectra of phycobilisome isolated form cells grown under green light (G) or under red light (H). Spectra of fraction 1 (black) and fraction 2 (orange) of NIES-3708 and fraction 1 (blue) and fraction 2 (purple) of NIES-3709 were normalized at maximum emission peaks. I, 77K fluorescence excitation spectra of fraction 1 isolated from strains NIES-3708 (thin dashed line) and NIES-3709 (thick solid line) grown under green light (green line) or under red light (red line). Spectra were normalized at APC peaks.

(*cpcF*, *cpcU*). Neither strain harbors genes for the rod linker for PC (*cpcC*), small rod-capping linker (*cpcD*), or core-membrane linker (*cpcL*). For biosynthesis of PE rods, which locate in peripheral position of PC rods, both strains harbor single copies of genes for the core subunit (*cpeBA*), phycoerythrobilin chromophore synthase (*pebBA*), and chromophore lyases (*cpeT*, *cpeS*). We found that the copy number of PE rod linker genes (*cpeC*, *cpeE*) is the only difference in composition of the phycobilisome genes between the two strains. Strain NIES-3708 harbors three copies of the PE rod linker gene (GM3708_1496, GM3708_1476, GM3708_1488), whereas NIES-3709 harbors four copies (GM3709_2916, GM3709_1229, GM3709_2683, GM3709_2915) (Fig. 3A). Phylogenetic analyses revealed that GM3708_1496, GM3708_1476, GM3709_2916, and GM3709_1229 belong to the *cpeC* family, whereas GM3709_2683, GM3709_2915, and GM3708_1488 belong to the *cpeE* family (Fig. 3B). Therefore, we designated GM3708_1476 and GM3709_1229 as *cpeC1*, GM3708_1496 and GM3709_2916 as *cpeC2*, GM3708_1488 and GM3709_2683 as *cpeE1*, and GM3709_2915 as *cpeE2* (Fig. 3A and B).

The CcaS/R photosensing system, which is composed of the green/red light–sensing cyanobacteriochrome CcaS and cognate response regulator CcaR, is conserved in the two strains.[18] Multiple

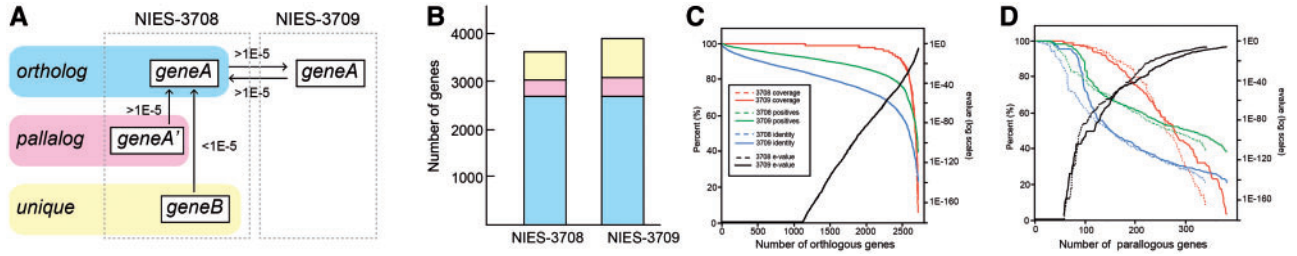

Figure 2. Bidirectional and unidirectional BLASTP best hit analyses. A, Method of assigning orthologs, paralogs, and unique genes using BLASTP. B, Numbers of orthologous, paralogous, and unique genes in strains NIES-3708 and NIES-3709. C and D, Ordered distribution of coverage, identity, similarity scores, and BLASTP e-values in the orthologous (C) and the paralogous genes (D).

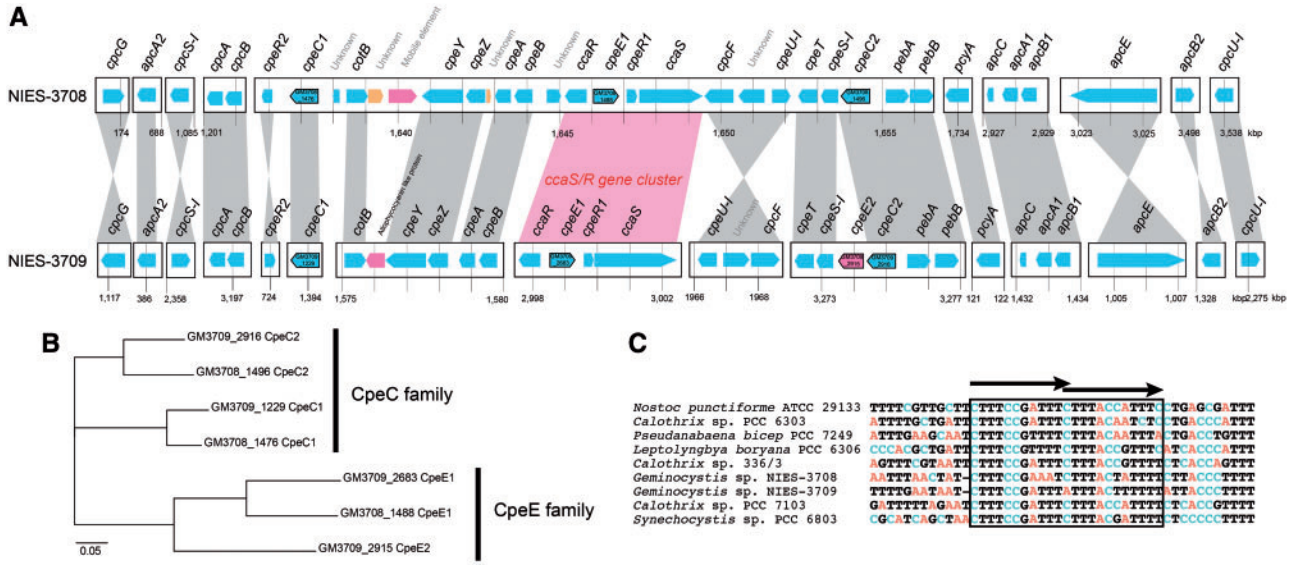

Figure 3. Gene organization of the phycobilisome and CcaS/R photosensing systems. A, Structure of APC, PC, PE, and CcaS/R system genes in strains NIES-3708 and NIES-3709. Orthologs (cyan), paralogs (pink), and unique (yellow) genes are shown. Numbers underneath the genes indicate the genome position. B, Phylogenetic tree of genes for the rod linker of PE. C, Alignment of CcaR binding motifs in the sequenced cyanobacteria strains. Arrows indicate the direct repeats of the G-box.[18]

alignment showed that the CcaR-binding direct repeat G-box is conserved in the promoter of the *cpeE1-cpeR1* operon in the two strains (Fig. 4C), suggesting that the CcaS/R system directly regulates the expression of this operon. CpeR has been established genetically, but not yet biochemically, to be the transcriptional activator for *cpeBA* and *pebAB*.[8,29,30] The CcaS/R system genes are clustered with the gene that encodes the rod-membrane linker CpcL in the typical CA2 performing cyanobacterium *N. punctiforme* but not in *Geminocystis* strains NIES-3708 and NIES-3709.[18] CpcL is a homolog of the rod-core linker protein CpcG, but it harbors a C-terminal hydrophobic helix that localizes it to the thylakoid membrane rather than the APC core.[31] In *Anabaena* sp. PCC 7120, CpcL has been shown to form atypical phycobilisome consisting of only rod to transfer light energy preferentially to the photosystem I.[5] The absence of *cpcL* in the two *Geminocystis* strains suggests that they harbor phycobilisomes with the typical core and rod structure and that they regulate PE, but not PC, using the CcaS/R system, which is distinct from CA2 of *Nostoc punctiforme*.[14,18]

Cyanobacteria harbor cyanobacteriochrome photosensors that bind a tetrapyrrole chromophore in the GAF (cGMP phosphodiesterase/adenylyl cyclase/FhlA) domain and photoconvert between two light-absorbing states.[32,33] Strains NIES-3708 and NIES-3709 harbor genes for eight cyanobacteriochromes (Fig. 4). We predicted the light wavelengths absorbed by these cyanobacteriochromes using the reference absorption spectra set of cyanobacteriochromes of *N. punctiforme* ATCC 29133,[34] an organism for which the spectral properties and sequence motifs that determine the absorption have been characterized most extensively. Seven of these cyanobacteriochromes are orthologs in the two strains: two blue/green-sensing phototaxis regulators of PixJ (GM3708_1098 and GM3708_1100, GM3709_2064, and GM3709_2062),[35,36] two violet sensors (GM3708_2600 and GM3708_34, GM3709_2442 and GM3709_2515), a violet-sensing circadian clock regulator of CikA (GM3708_1578, GM3709_546),[37] a green/red-sensing CA regulator of CcaS (GM3708_1490, GM3709_2685)[14] and a red/green sensor (GM3708_2773, GM3709_1938). The protochromic triad residues required for the green/red photocycle is conserved in CcaS of strains NIES-3708 and NIES-3709[15] (Supplementary Fig. S4). For unique cyanobacteriochrome genes, strain NIES-3708 harbors the gene for a blue/green sensor (GM3708_1188), whereas strain NIES-3709 harbors the gene for a different blue/green sensor (GM3709_3634). In CA3 of *F. diplosiphon*, the orange/teal light–sensing cyanobacteriochrome DpxA is suggested to support PE repression in addition to RcaE.[38] No gene encoding DpxA or RcaE was found in the genomes

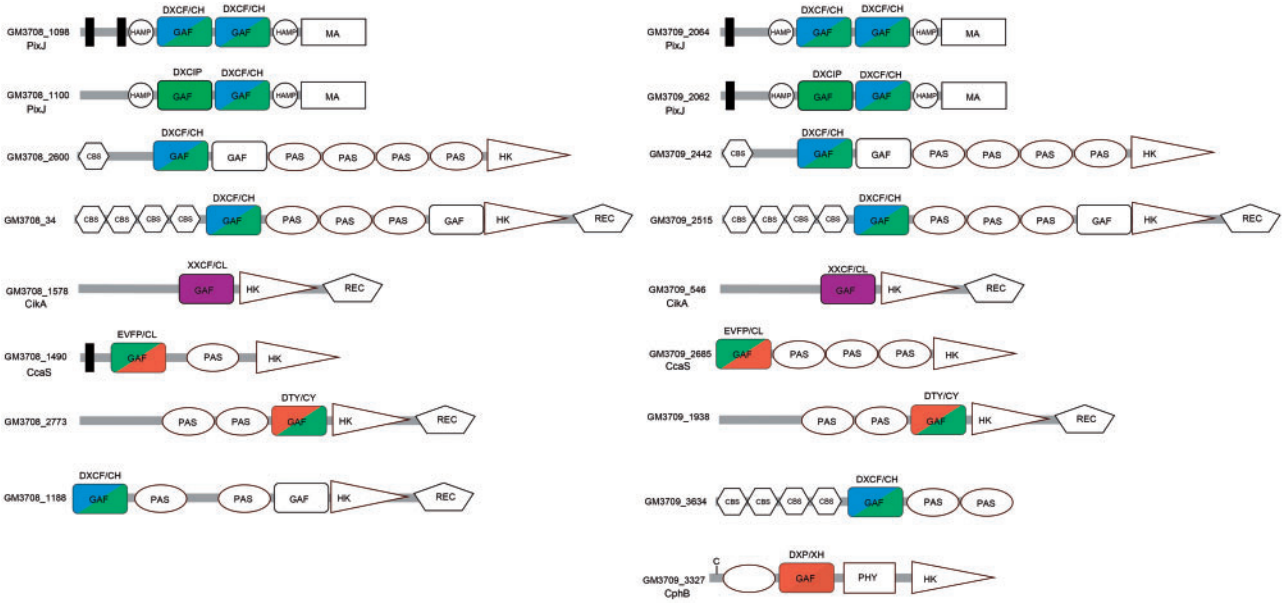

Figure 4. Domain architecture of cyanobacteriochromes and phytochromes in strains NIES-3708 and NIES-3709. GAF, GAF domain; PAS, PAS domain; HAMP, HAMP domain; HK, histidine kinase domain; REC, receiver domain; CBS, CBS domain. The predicted two light-absorbing colors of the chromophore-binding GAF domain are shown. Predicted transmembrane regions were shown as black box. Conserved sequence motif in the bilin-binding pocket of each photosensing GAF domain (Supplementary Figs. S3 and S4) are shown.

of strains NIES-3708 and NIES-3709, suggesting that the CcaS/R system is the sole CA regulator in these strains. We found that strain NIES-3708 displayed negative phototaxis for red light but not for green light, whereas strain NIES-3709 did not show phototaxis (data not shown). NIES-3708 harbours phototaxis regulators of the PixJ class but not of the UirS/PixA or Cph2 class,[39–41] implying that PixJ may regulate the phototaxis of NIES-3708. NIES-3709 also harbours the red/far-red sensor of the biliverdin-binding phytochrome CphB (GM3709_3327),[42] suggesting that this strain responds to near far-red light (700–750 nm). Further studies are necessary to delineate these complex light-sensing pathways.

3.3. Transcriptome changes

Changes in the global gene expression during CA3 have been studied in *F. diplosiphon* by genomic microarray analysis,[43] but expression changes during CA2 have not been explored. Therefore, we performed RNA-Seq of strains NIES-3708 and NIES-3709 during their acclimation to green or red light (Fig. 5). Numbers of raw mapped reads and normalized RPKM values in triplicate were determined for all CDSs (Supplementary Tables S3 and S4). We searched for genes that have been annotated with known functions and that showed a >2-fold change in expression between green and red light (Fig. 5). In strain NIES-3708 under red light, gene expression levels were high for photosystem II (*psb* genes), photosystem I (*psa* genes), ATPase (*atp* genes), NDH (*ndh* genes), iron transporting systems (*fecC*, *exbBD*, *futA1*, *piuC*, *feoAB*), and photoprotection protein during iron-starvation (*isiAB*); under green light, expression levels were high for PE components (*cpeBA*, *cpeYZ*, *ccaS*, *pebAB*) and type IV pili (*pilA1*, *pilA2*, *pilY1*). In strain NIES-3709 under red light, gene expression levels were high for nitrate transport system (*nrtA*, *nrtC*) and photoprotection protein (*isiA*); under green light, expression levels were high for PE (*cpeBA*, *cpeYZ*, *ccaS*, *pebAB*), photosystem II (*psbA*), cytochrome C553 subunit, coproporphyrinogen III oxidase (*cpoX*), and sigma factor (*sigG*). In an RNA-Seq study of *Leptolyngbya* sp. strain JSC-1 during iron starvation, the gene expression of the most components of the photosynthetic apparatus did not change.[44] Therefore, we speculate that the higher synthesis activity of photosynthetic apparatus components requiring iron would produce relatively iron-limited conditions under red light for strain NIES-3708, leading to induction of iron uptake and iron-starvation resistance systems. PilA is a component of type IV pili and is required for biofilm formation, uptake of DNA, and phototaxis.[45] The *pilA1* of strain NIES-3708 showed the highest expression (RPKM of 276,643 under green light, 68,645 under red light) and was induced by 4-fold under green light, whereas *pilA* of strain NIES-3709 was not induced (Fig. 5; Supplementary Tables S3 and S4). It will be interesting to explore whether the pili genes of strain NIES-3708 is regulated by a cyanobacteriochrome-class photoreceptor (Fig. 4).

We then carefully compared the expression of phycobilisome components. The order of RPKM values for the phycobilisome genes were similar in the two strains: $\sim 10^4$ for major phycobiliproteins, $\sim 10^3$ for linker proteins, and 10^1 to 10^3 for regulators, bilin synthases, and bilin lyases (Fig. 6). In both NIES-3708 and NIES-3709, expression of genes for the APC core and PC rods was greater under red light than green light. For PE, genes for phycobiliproteins (*cpeBA*), one rod linker (*cpeE1*), one regulator (*cpeR1*), phycoerythrobilin synthase (*pebAB*), and bilin lyase (*cpeYZ*) were significantly induced under green light in the two strains (Fig. 6). In contrast, expression of genes for other PE rod linkers (*cpeE2*, *cpeC1*, *cpeC2*) and bilin lyases (*cpeSUT*) were induced to a similar or higher degree under red light. Our genome analysis of the two strains suggested that the CcaS/R photosensing system directly regulates the expression of *cpeE1* and *cpeR1*, and the induced CpeR1 regulator further activates the expression of *cpeBA*, *cpeYZ*, and *pebAB*[29,46]. In *N. punctiforme*, higher expression of *cpcBA*, *cpcG*, and *cpeE* under red light than green light has been observed in both *ccaS* and *ccaR* mutants, suggesting that the system for balancing excitation between phycobilisome and photosystems is responsible for the red-light

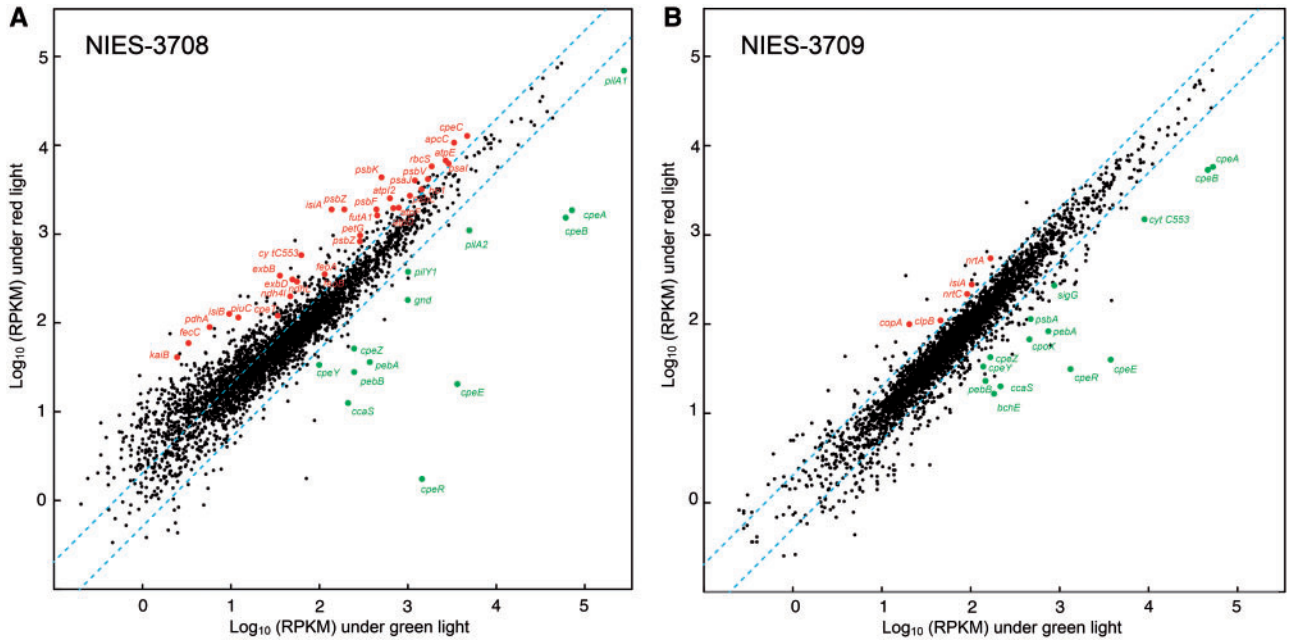

Figure 5. Transcriptome comparison during CA. RNA-Seq analysis of strains NIES-3708 (left) and NIES-3709 (right) under green or red light. Dashed cyan lines indicate the borders of 2-fold change in expression.

activation of these genes[1]. We assume that the same balancing system activates the expression of genes for phycobilisome components other than *cpeE1*, *cpeR1*, *cpeBA*, *cpeYZ*, and *pebAB* in strains NIES-3708 and NIES-3709. Notably, the linker genes expressed under green light were *cpeC1*, *cpeC2*, *cpeE1*, and *cpeE2* for strain NIES-3709 and *cpeC1*, *cpeC2*, and *cpeE1* for strain NIES-3708. In the model cyanobacterium *Synechocystis* sp. PCC 6803, deletion of *cpcC1* causes absence of not only CpcC1 but also CpcC2 in the phycobilisome,[47] suggesting that CpcC1 is located in a central position in the PC rod whereas CpcC2 is located in a peripheral position. In this study, expression of CpeE1 is strictly regulated by the CcaS/R system, suggesting that the role of CpeE1 differs that of other rod linkers. We speculate that in strains NIES-3708 and NIES-3709, the light-regulated CpeE1 is located at the base position of PE rods to control energy transfer from PE to PC. Taken our data of spectroscopy, genome and transcriptome together, we assume the molecular process of the CA in strains NIES-3708 and NIES-3709 as follows. Both strains harbor a canonical phycobilisome rod and core structure. The CcaS/R photosystem increases the 'length' of PE rods on the PC rods under green light, whereas the excitation balancing system of phycobilisome and photosystems increases the 'number' of phycobilisomes containing only the PC rods and APC core under red light. The copy number of PE rod linkers differentiated the length of PE rods between the two strains, leading to the different cellular PE content under green light. Further analyses of the phycobilisome of the two strains are required to confirm the exact location of each rod linker.

We observed that expression of the gene for the CcaS photosensor was induced under green light: 13.3-fold in strain NIES-3708 and 9.4-fold in NIES-3709. On the other hand, expression of the gene for the cognate response regulator CcaR was not induced under green light (1.0-fold difference in strain NIES-3708, and 0.7-fold in NIES-3709). When CcaS/R is heterologously expressed in *Escherichia coli*, green light-induced P_{cpcG2} activity is enhanced proportional to the increase in CcaS protein.[48] Consistently, *ccaS* is located in the green light-induced operon in all other CA2 species (Fig. 7), suggesting that the induction of *ccaS* is a common mechanism to improve the induction ratio of phycobilisome genes. The induction ratios of *cpeE1* and *cpeR1* were 799 and 177 in strain NIES-3708 and 97 and 42 in NIES-3709, respectively. Thus, the CcaS/R system of NIES-3708 shows higher induction than that of NIES-3709 (Supplementary Tables S3 and S4). The different induction ratios of *cpeR1* led to a 39- and 9-fold induction of *cpeBA* in strains NIES-3708 and NIES-3709, respectively (Supplementary Tables S3 and S4), leading to the observed difference in repression activity of PE under red light (Fig. 1E). Because the expression of each of *ccaS* and *ccaR* was similar in the two strains, we assume that changes in the kinase or phosphatase activity of the CcaS/R system caused the differences in PE-repression activity. The CcaS/R system is emerging as a useful photoswitch in optogenetics. Screening for the best photoswitch genes in the emerging cyanobacterial genomes will be important for the development of superior optogenetic tools.

3.4. Reclassification of CA

In the 1970s, based on the changes of absorption peaks of partially purified phycobiliproteins, two types of CA were characterized: CA2 modulates PE content only, whereas CA3 modulates both PE and PC contents. Therefore, CA2 and CA3 have been defined only in cyanobacteria that produce both PC and PE. However, this spectrum-based classification of CA does not entirely fit with the diverse molecular processes of CA, which have been revealed by recent genome-based studies. First, the CcaS/R photosensing system for CA2 is also found in cyanobacteria that produce PC but not PE, such as *Synechocystis* sp. PCC 6803, *Leptolyngbya boryana* PCC 6306, *Pseudanabaena bicep* PCC 7429, and *Anabaena* sp. 39865 (Fig. 7). Regulation of *cpcL* in these species is suggested to maintain the

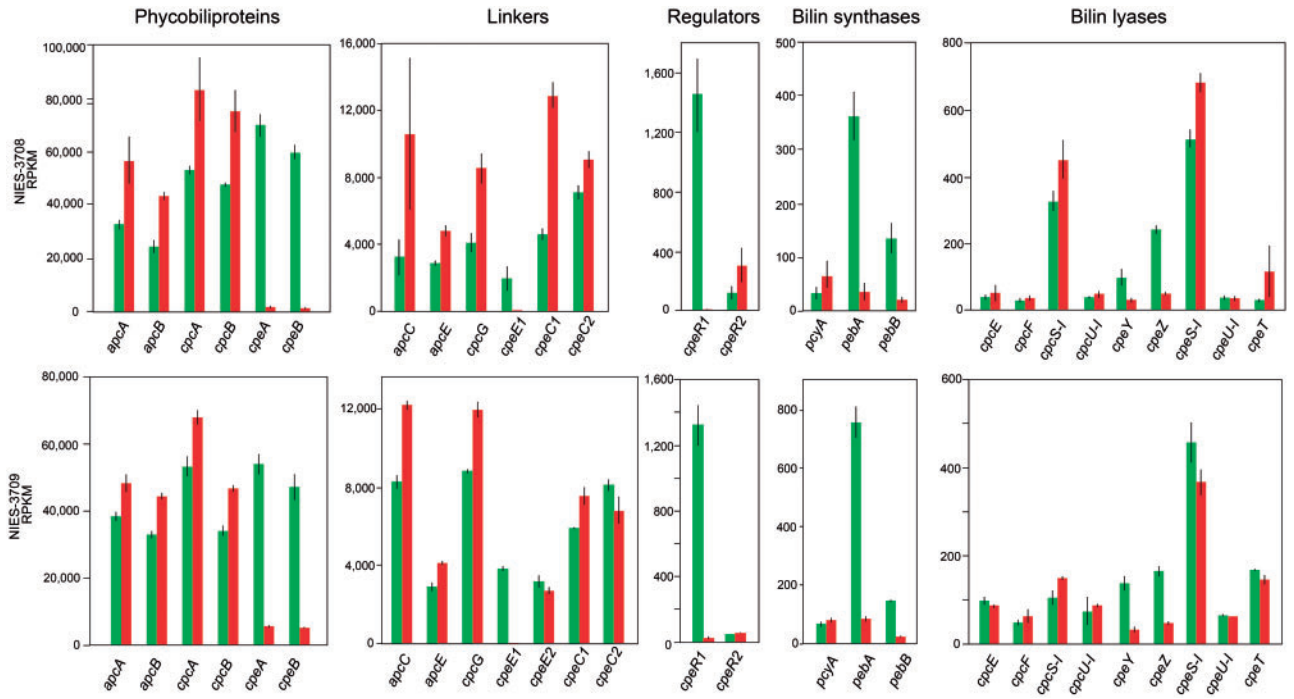

Figure 6. RPKM values for phycobilisome and related genes. RPKM values for genes encoding phycobiliproteins, linkers, regulators bilin synthases, and bilin lyases genes in strains NIES-3708 (upper) and NIES-3709 (lower) under green (green, left bar) or red (red, right bar) light. Error bars indicate standard deviation of triplicate measurements.

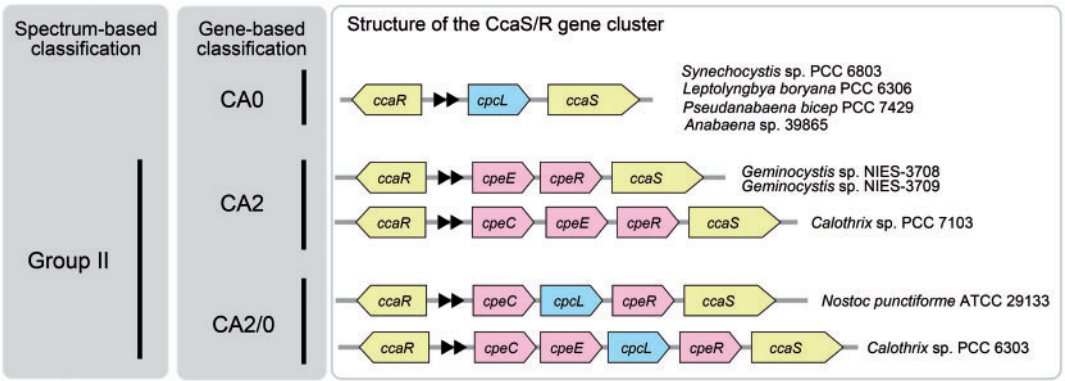

Figure 7. CA types of strains NIES-3708 and NIES-3709. Conventional (spectrum-based) classification of CA and the genome-based classification proposed in this study. Gene structures of the photosensing CcaS/R system (yellow), phycoerythrin (pink) and phycocyanin (cyan) is shown.

excitation balance between photosystems I and II.[14,18] Second, the typical CA2-performing cyanobacterium *N. punctiforme* regulates both PE genes and *cpcL* using the CcaS/R system (Fig. 7). This indicates that *N. punctiforme* regulates both PE and PC content and apparently conflicts with the initial definition of CA2. In addition, standardization of light quality, light intensity, incubation conditions, and extraction of phycobiliproteins among laboratories is difficult. For example, *Calothrix* sp. PCC 7103 is classified as a Group III strain, but it harbours the CcaS/R system in the genome. To resolve these inconsistencies, we propose to reclassify the conventional CA2 based on the gene sets in the CcaS/R-photosensing cluster, including the cyanobacterial species that do not harbor PE (Fig. 7). In this reclassification, the *cpcL* regulation in cyanobacteria that produce only PC is designated CA of type zero (CA0). The regulation of only PE genes is designated as genuine CA2, as identified in our present study. The regulation of PE genes and *cpcL* is designated as a CA2/0 hybrid. This genome-based classification of CA will be useful as much more cyanobacterial genomes will be available in the near future.

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Conflict of interest

None declared.

Supplementary data

Supplementary data are available at *DNARES* Online.

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