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PLANT & CELL PHYSIOLOGY

# In Vitro Enzymatic Activity Assays Implicate the Existence of the Chlorophyll Cycle in Chlorophyll *b*-Containing Cyanobacteria

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(Received April 11, 2019; Accepted August 2, 2019)

**In plants, chlorophyll (Chl) *a* and *b* are interconvertible by the action of three enzymes—chlorophyllide *a* oxygenase, Chl *b* reductase (CBR) and 7-hydroxymethyl chlorophyll *a* reductase (HCAR). These reactions are collectively referred to as the Chl cycle. In plants, this cyclic pathway ubiquitously exists and plays essential roles in acclimation to different light conditions at various developmental stages. By contrast, only a limited number of cyanobacteria species produce Chl *b*, and these include *Prochlorococcus*, *Prochloron*, *Prochlorothrix* and *Acaryochloris*. In this study, we investigated a possible existence of the Chl cycle in Chl *b* synthesizing cyanobacteria by testing in vitro enzymatic activities of CBR and HCAR homologs from *Prochlorothrix hollandica* and *Acaryochloris* RCC1774. All of these proteins show respective CBR and HCAR activity in vitro, indicating that both cyanobacteria possess the potential to complete the Chl cycle. It is also found that CBR and HCAR orthologs are distributed only in the Chl *b*-containing cyanobacteria that habitat shallow seas or freshwater, where light conditions change dynamically, whereas they are not found in *Prochlorococcus* species that usually habitat environments with fixed lighting. Taken together, our results implicate a possibility that the Chl cycle functions for light acclimation in Chl *b*-containing cyanobacteria.**

**Keywords:** Chlorophyll • Cyanobacterium • Enzyme • Evolution • Promiscuous activity.

## Introduction

Chlorophyll (Chl) plays crucial roles in photosynthesis by harvesting light energy and driving electron transfer (Renger and Schlodder 2010). Photosynthetic organisms have to change their light-harvesting capacity and number of photosystems to perform efficient and safe photosynthesis under changing light environments (Webb and Melis 1995, Dietzel et al. 2008). To achieve these photosystem restructurings, synthesis and degradation of Chl must be finely regulated in response to light environments (Tanaka et al. 2001, Masuda et al. 2003). Green plants contain Chl *a* and Chl *b*, which have different absorbance spectra and contribute to the use of a wide range of light spectra (Chen 2014). Chl *a* is responsible for charge separation in photosynthetic reaction centers and is a major light-harvesting pigment both in the core (CP43/47 of photosystem II and PsaA/B of photosystem I) and the peripheral antenna complexes. By contrast, Chl *b* is responsible only for light harvesting. The localization of Chl *b* in light-harvesting systems is different among green plants (Kunugi et al. 2016). Photosynthetic organisms adapted to high light levels, such as Chlamydomonas and land plants, contain Chl *b* only in the peripheral antenna complexes, whereas Chl *b* exists in both the core and the peripheral antenna complexes in green algae living in deep seas. The green plants alter their antenna size by changing the Chl *a*/*b* ratio (Bailey et al. 2001). When the plants grow under low light conditions, the plants have a low Chl *a*/*b* ratio and large antenna size. Therefore, the Chl *a*/*b* ratio must be regulated in response to changes in the light environment. Green plants employ various strategies to regulate the Chl *a*/*b* ratio. Chlorophyllide (Chlide) *a* oxygenase (CAO) is responsible for Chl *b* synthesis. CAO protein levels are finely regulated at the transcriptional level and protein degradation rates (Tanaka and Tanaka 2005, Yamasato et al. 2005, Nakagawara et al. 2007). Another important reaction for regulating the Chl *a*/*b* ratio is the conversion of Chl *b* to Chl *a* (Tanaka and Tanaka 2007). In this pathway, Chl *b* is converted to 7-hydroxymethyl chlorophyll *a* (HMChl *a*) by Chl *b* reductase (CBR; Kusaba et al. 2007), followed by the reduction to Chl *a* by HMChl *a* reductase [7-hydroxymethyl chlorophyll *a* reductase (HCAR); Meguro et al. 2011; **Fig. 1**]. This pathway is responsible not only for regulation of the Chl *a*/*b* ratio but also for the degradation of Chl *b* during senescence because Chl *b* must be converted to Chl *a* before degradation (Hörtensteiner et al. 1995). Therefore, interconversion of Chl *a* and Chl *b* (the Chl cycle) plays a crucial role in various developmental stages in green plants.

Chl *b* is not present as free pigment but as light-harvesting Chl *a*/*b*-protein complexes (LHC) in the chloroplast. Mutant analysis with *Arabidopsis* (Horie et al. 2009) and rice (Kusaba et al. 2007) demonstrated that LHC is not degraded in the CBR mutant during senescence. In vitro experiments indicated that Chl *b* in LHC can be a substrate of recombinant CBR (Horie et al. 2009). These experiments indicate that Chl *b* in LHC is the primary substrate of CBR in chloroplasts. CBR might have evolved to be able to catalyze Chl *b* in LHC.

Chl *b* is found not only in eukaryotic green plants but also in some cyanobacterial lineages, such as *Prochlorococcus*,

*Plant Cell Physiol.* 0(0): 1–12 (2019) doi:10.1093/pcp/pcz157, Advance Access publication on 8 August 2019, available online at https://academic.oup.com/pcp

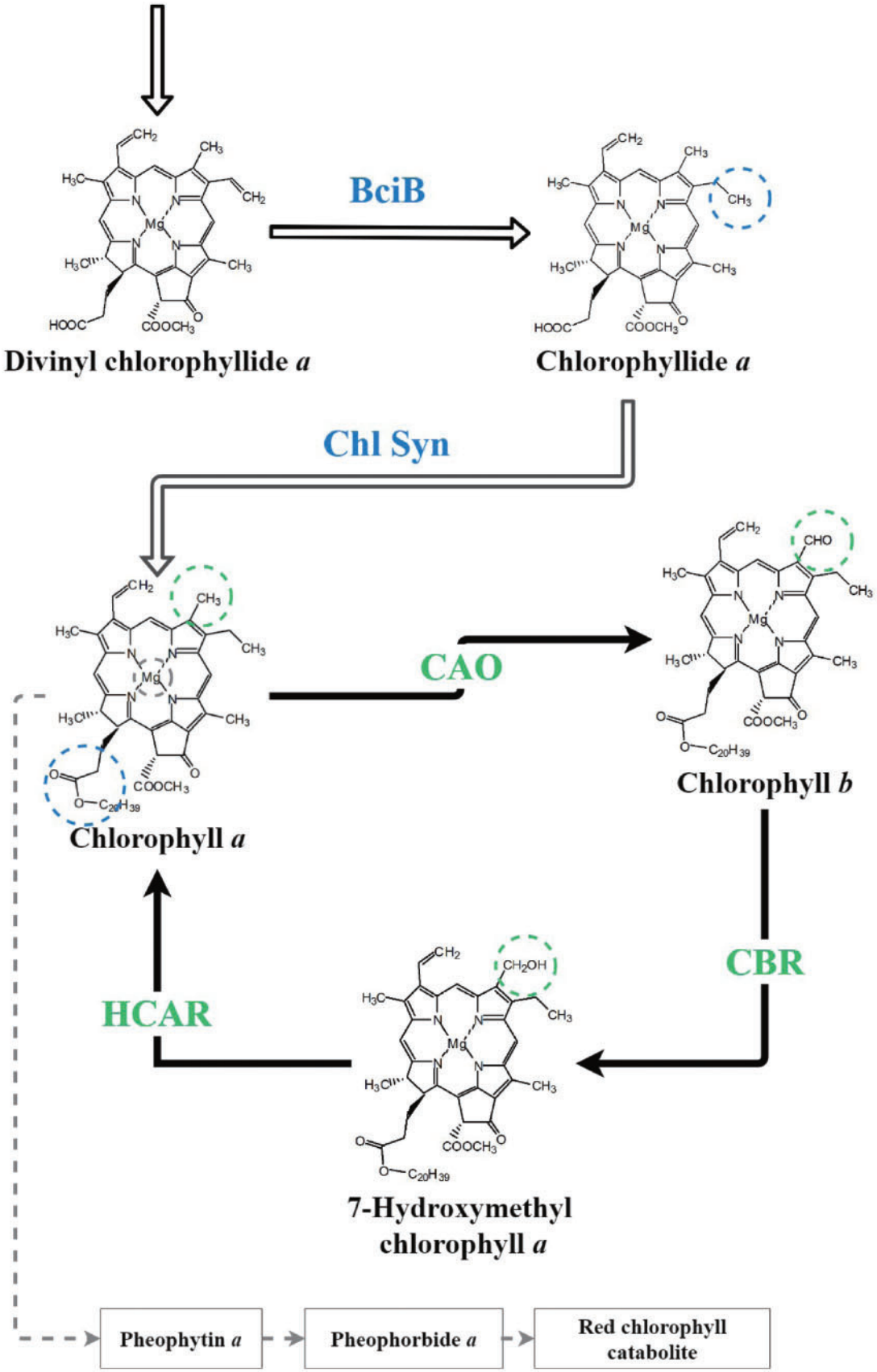


**Fig. 1** Chl metabolic pathway. Outline arrow, black arrow and dashed arrow represent the Chl synthesis pathway, Chl cycle and Chl degradation pathway, respectively. Dashed circle indicates the reaction site. Chl Syn, Chl synthase.

*Prochloron* and *Prochlorothrix* (Palenik and Haselkorn 1992). Recently, a novel species of *Acaryochloris* was found that has Chl *b* instead of Chl *d* (Partensky et al. 2018). These organisms containing Chl *b* do not form a single cluster in the phylogenetic tree but appear independently in the cyanobacterial lineage (Partensky et al. 2018). Cyanobacteria do not have LHC. Instead, Chl *b* is incorporated into the prochlorophyte Chl *b*-binding protein (PCB; Bibby et al. 2003, Bumba et al. 2005), which is not phylogenetically related to LHC (La Roche et al. 1996). The localization of Chl *b* in *Acaryochloris* has not been reported, but it might be present in PCB, as in *Prochlorococcus* and *Prochlorothrix*.

Chl metabolic pathways, including biosynthesis, the Chl cycle and degradation, have been determined (Tanaka and Tanaka 2005, Hörtensteiner 2006, Nagata et al. 2007), and major enzymes for these pathways have been identified in land plants (Nagata et al. 2005, Hauenstein et al. 2016, Shimoda et al. 2016). Pathway and enzymes of Chl biosynthesis are also determined in cyanobacteria. Although Chl degradation is an important process for cyanobacteria, the enzymes and pathway of Chl degradation have not been determined. As for the Chl cycle, Chl *b* is synthesized by CAO in cyanobacteria, as in green plants (Satoh and Tanaka 2006). However, it is not evident whether these cyanobacteria have a Chl *b*-to-*a* conversion pathway because CBR and HCAR of cyanobacteria could not be proposed only by the sequence similarity and phylogenetic tree.

In this study, we constructed phylogenetic trees of CBR, HCAR and their homologous genes and determined the candidates of these genes in *Prochlorothrix hollandica* and *Acaryochloris* RCC1774. Then, we tried to detect the enzymatic activities using the recombinant proteins of these candidate genes and found the CBR and HCAR activities, implicating the presence of the Chl cycle in cyanobacteria containing Chl *b*. We discuss the functions and evolution of the Chl cycle in cyanobacteria.

## Results

### Pigment composition of *P. hollandica* and *Acaryochloris* RCC1774

Photosynthetic pigments of *P. hollandica* and *Acaryochloris* RCC1774 were examined by HPLC (**Fig. 2A**). Pigment compositions were similar to those in previous reports (Takaichi et al. 2012, Partensky et al. 2018). The elution profiles of HPLC were similar between the two organisms with some differences. *Acaryochloris* RCC1774 and *P. hollandica* have $\alpha$- and $\beta$-carotene, respectively. Both of them have $\epsilon,\epsilon$-carotene (**Fig. 2B**). Chl *a*/*b* ratios of *Acaryochloris* RCC1774 and *P. hollandica* were 6.8 and 13.5, respectively. *Acaryochloris* RCC1774 accumulates divinyl protochlorophyllide *a*, which is generally a precursor of Chl biosynthesis, whereas *P. hollandica* does not accumulate this pigment. The absorbance spectrum of this pigment is similar to that of Chl *c*; therefore, it may function for light harvesting in *Acaryochloris* RCC1774 like it does in *Prochloron* (Larkum et al. 1994).

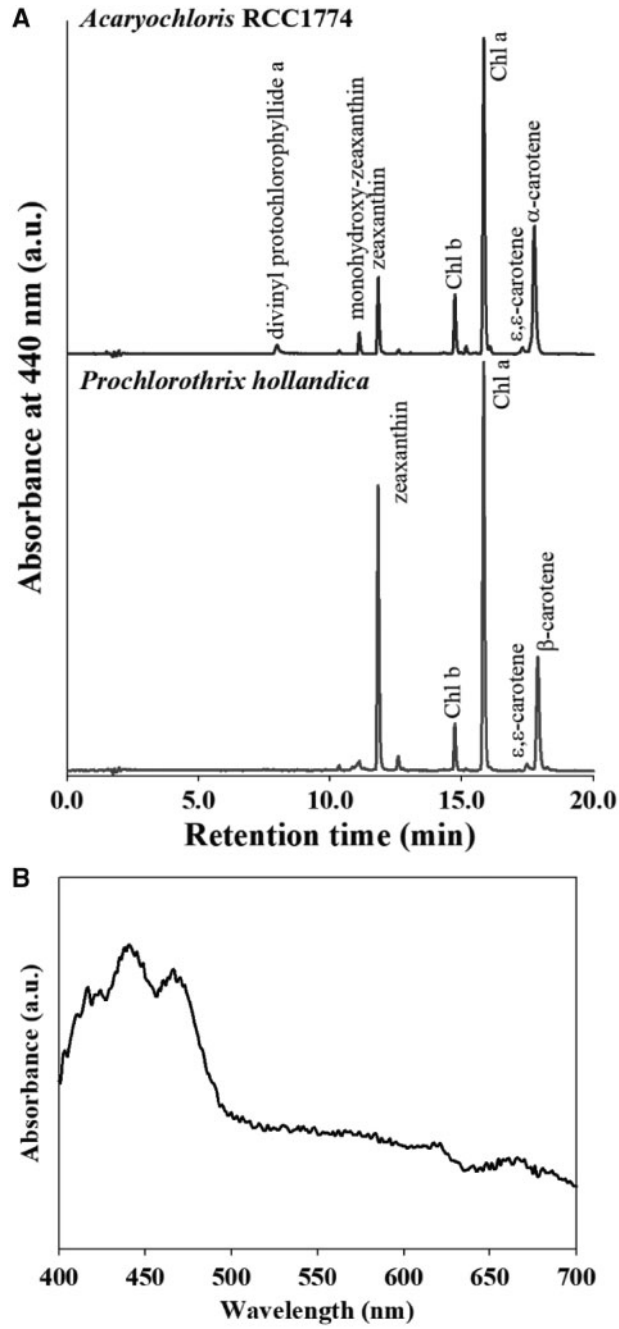


**Fig. 2** Pigment compositions of *Acaryochloris* RCC1774 and *P. hollandica*. (A) HPLC profiles of the pigment monitored at 440 nm. The peaks were identified by their retention time and spectrum. (B) The absorption spectrum of $\epsilon,\epsilon$-carotene detected in *Acaryochloris* RCC1774.

### Phylogenetic analysis of the Chl cycle enzymes

CAO catalyzes the oxidation of a methyl group on Chl *a* to a formyl group (Oster et al. 2000), and it is responsible for the formation of Chl *b* in all organisms containing Chl *b* (Tomitani et al. 1999). The phylogenetic tree of CAO (Supplementary Fig. S1) demonstrates the phylogenetic relationship of these organisms. The *CAO* gene of green plants

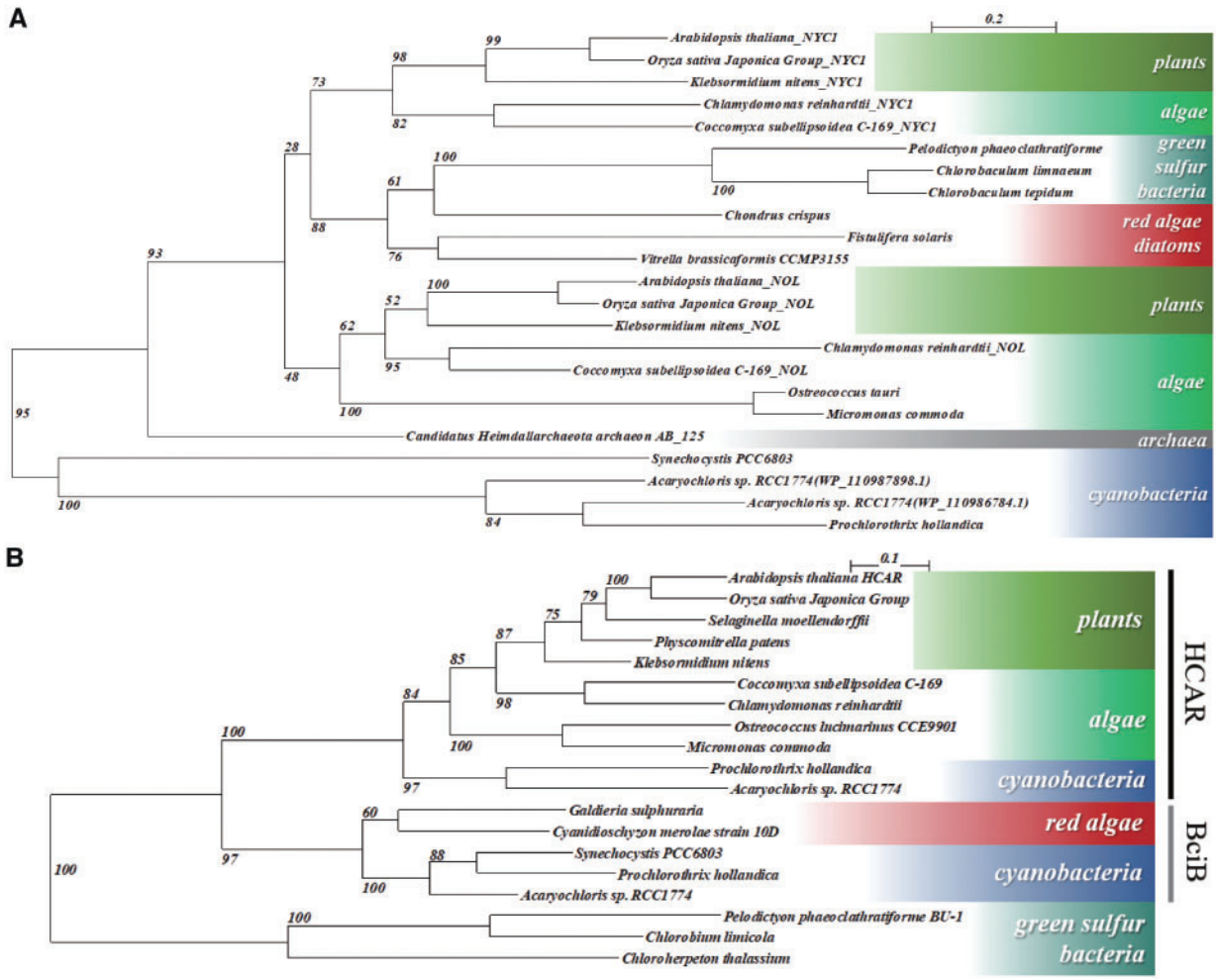


**Fig. 3** Phylogenetic trees of CBR, HCAR and BciB. The phylogenetic trees were constructed by maximum likelihood. The numbers at each node represent the bootstrap value and the number of amino acid substitutions per site is illustrated by the scale bar. (A) Phylogenetic tree of CBR. *Arabidopsis* NYC1 (AT4G13250); *Arabidopsis* NOL (AT5G04900); *Oryza sativa Japonica* Group NOL (XP_015628274.1); *O. sativa Japonica* Group NYC1 (XP_015621887.1); *Klebsormidium nitens* NYC1 (GAQ77737.1); *K. nitens* NOL (GAQ87774.1); *Coccomyxa subellipsoidea* C-169 NYC1 (XP_005652224.1); *C. subellipsoidea* C-169 NOL (XP_005646276.1); *Pelodictyon phaeoclathratiforme* (WP_012508106.1); *Ostreococcus lucimarinus* CCE9901(XP_001415854.1 ); *Micromonas commoda* (XP_002508859.1); *Chlamydomonas reinhardtii* NYC1 (XP_001697080.1); *C. reinhardtii* NOL (XP_001701347.1); *Vitrella brassicaformis* CCMP3155 (CEM24690.1); *Candidatus Heimdallarchaeota archaeon* AB_125 (OLS31532.1); *Chondrus crispus* (XP_005716045.1); *Chlorobaculum tepidum* (WP_010932815.1); *Chlorobaculum limnaeum* (WP_069809375.1); *Fistulifera solaris* (GAX23003.1); *P. hollandica* (WP_081599361.1); *Acaryochloris* RCC1774 (1) (WP_110987898.1); *Acaryochloris* RCC1774 (2) (WP_110986784.1); *Synechocystis* PCC6803 (WP_041428273.1). (B) Phylogenetic tree of BciB and HCAR. *C. reinhardtii* (PNW76723.1); *M. commoda* (XP_002503439.1); *O. lucimarinus* CCE9901 (XP_001416225.1); *C. subellipsoidea* C-169 (XP_005648937.1); *K. nitens* (GAQ88093.1); *Physcomitrella patens* (XP_024368500.1); *Selaginella moellendorffii* (XP_024538910.1); *O. sativa Japonica* Group (XP_015636785.1); *Arabidopsis* HCAR (AT1G04620.1); *Acaryochloris* RCC1774 HCAR (WP_110987361.1); *P. hollandica* HCAR (WP_017711629.1); *Acaryochloris* RCC1774 BciB (PZD72398.1); *P. hollandica* BciB (WP_044076442.1); *Synechocystis* PCC6803 (WP_010873198.1); *Cyanidioschyzon merolae* strain 10D (XP_005534820.1); *Galdieria sulphuraria* (XP_005706147.1); *Chloroherpeton thalassium* (WP_012499756.1); *Chlorobium limicola* (WP_059139293.1); *P. phaeoclathratiforme BU-1* (ACF44893.1).

and cyanobacteria shares the common ancestor suggesting that the *CAO* gene has been transferred from an ancestral cyanobacterium to plants via the endosymbiosis event.

CBR catalyzes the reduction of a formyl group on Chl *b* to a hydroxymethyl group, which is the first step of Chl *b*-to-*a* conversion. CBR belongs to a short-chain dehydrogenase family that has an enormous number of members and is greatly diversified (Kallberg et al. 2002). CBR homologs are widely distributed not only in green plants but also in other organisms, including red algae, diatoms, cyanobacteria and photosynthetic bacteria (**Fig. 3A**). Green plants have two CBRs, Non-Yellow Coloring 1 (NYC1) and NYC1-Like (NOL), whereas a group of green algae, Pracinophytes (*Ostreococcus* and *Micromonas*), has only one *CBR* gene. The phylogenetic analysis did not clearly show to which group *Ostreococcus* and *Micromonas* CBRs belong. These CBRs have no membrane-spanning helix, which is a characteristic feature of NOL, suggesting that they belong to the NOL members.

CBR homologs of red algae and diatoms are most closely related to green plant CBRs phylogenetically, although they do not produce Chl *b*. Interestingly, green sulfur bacteria also have CBR homologs. Genes highly homologous to CBR were found only in photosynthetic organisms, suggesting that homologous genes are related to photosynthesis. CBR homologs of *P. hollandica* and *Acaryochloris* RCC1774 were most distantly related to green plant CBRs phylogenetically in this tree and formed a cluster with other cyanobacterial CBR homologs (**Fig. 3A**), although these two cyanobacteria have Chl *b*. This distribution profile of cyanobacterial CBR homologs does not indicate whether they are genuine orthologs that encode functional CBR or not.

In the Chl cycle, HMChl *a* is converted to Chl *a* by HCAR. We retrieved its homologous genes using *Arabidopsis* HCAR (AT1G04620) as a query. Many homologous proteins were found in photosynthetic eukaryotes and cyanobacteria. The phylogenetic tree of these proteins was separated into two clusters, one is HCAR and the other is 8-vinyl reductase (BciB, also known as F-DVR; Ito et al. 2008, Liu and Bryant 2011). Green plants have HCAR but not BciB because they employ BciA (also known as N-DVR) instead of BciB. Cyanobacteria use BciB instead of BciA and most of them have neither Chl *b* nor HCAR. However, cyanobacteria containing Chl *b*, *P. hollandica* and *Acaryochloris* RCC1774, have two homologous genes, one of which belongs to the HCAR cluster and the other to the BciB cluster (**Fig. 3B**). This phylogenetic analysis implicates that *P. hollandica* and *Acaryochloris* RCC1774 have HCAR.

## Enzymatic analysis of the candidate genes of *P. hollandica* and *Acaryochloris* RCC1774

To clarify whether CBR homologs of *P. hollandica* and *Acaryochloris* RCC1774 have CBR activity or not, the recombinant proteins encoded by these genes were expressed in *Escherichia coli*. Immunoblotting analysis showed that these proteins were successfully expressed in *E. coli* and found in a soluble fraction (**Fig. 4**).

In *P. hollandica*, we found one candidate gene, WP_081599361, for a potential CBR gene by phylogenetic analysis. When the recombinant protein of WP_081599361 was incubated with Chl *b*, we detected two new peaks in the HPLC chromatograms for pigment analysis (**Fig. 5A**, line 5), neither of which were found in the negative control experiment (**Fig. 5A**, line 4). The retention times and absorption spectra of Peak 1 and Peak 2 matched those of HMChl *a* and its C13-2 epimer form, i.e. HMChl *a*′ (**Fig. 5B**). The activity was diminished by the depletion of NADPH (**Fig. 5A**, line 6) indicating that the protein uses NADPH as a reductant like *Arabidopsis* CBR.

*Acaryochloris* RCC1774 has two candidates, WP_110987898 and WP_110986784, for a potential CBR gene. It should be noted that the PZD74811 locus encodes a peptide sequence similar to the second half of the CBR sequences but it appears to lack the first half. Therefore, we did not include this gene for further analysis. We examined the recombinant protein of WP_110986784 activity and found its CBR activity (**Fig 5A**, line 7). When NADPH was omitted from the assay mixture, these two peaks did not appear (**Fig. 5A**, line 8), which indicate that the appearance of these peaks is dependent on enzymatic activity. Taken together, we concluded that the gene product of *Acaryochloris* RCC1773 WP_110986784 has CBR activity. By contrast, we could not detect CBR activity with the gene product of WP_110987898.

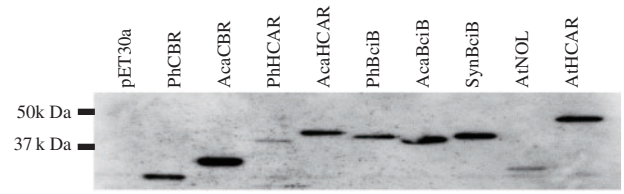


**Fig. 4** Immunoblotting analysis of recombinant proteins using a specific antibody against the histidine tag. Candidate genes of CBR, HCAR or BciB were expressed in *E. coli* and soluble fraction of the cell lysate was analyzed by immunoblotting. The markers for molecular size are shown in the left side. The cell lysate of *E. coli* having an empty vector (pET30a) was employed for the negative standard. PhCBR, *P. hollandica* CBR; AcaCBR, *Acaryochloris* RCC1774 CBR; PhHCAR, *P. hollandica* HCAR; AcaHCAR, *Acaryochloris* RCC1774 HCAR; PhBciB, *P. hollandica* BciB; AcaBciB, *Acaryochloris* RCC1774 BciB; SynBciB, Synechocystis BciB; AtNOL, *Arabidopsis* NOL; AtHCAR, *Arabidopsis* HCAR.

These results indicate that *P. hollandica* and *Acaryochloris* RCC1774 have CBR orthologs. A significant amount of the epimer type of HMChl *a* was formed after incubation of Chl *a* with *P. hollandica* and *Acaryochloris* RCC1774 CBRs (**Fig. 5A**). By contrast, HPLC analysis showed that Chl *a*′ (an epimer of Chl *a*) was not accumulated in a large amount in cells (**Fig. 2**). We speculate that the production of this epimer could be derived from Chl *b*′ produced by incubation with *E. coli* lysate, and then it was converted to HMChl *a*′ by CBR. Alternatively, the discrepancy could be explained by assuming some mechanism that suppresses the CBR-mediated formation of HMChl *a*′ in the cell.

Phylogenetic analysis suggests that *P. hollandica* WP_017711629 and *Acaryochloris* RCC1774 WP_110987361 are HCARs. Recombinant proteins of these genes were expressed in *E. coli*, and the cell extract was incubated with HMChl *a*. HPLC analysis showed that HMChl *a* was converted to Chl *a* by these proteins (**Fig. 6A**, line 6 and line 8). The production of Chl *a* was also confirmed by the detection of the pigment by fluorescence at 680 nm with excitation at 430 nm (Supplementary Fig. S2). These results indicate that the gene products of *P. hollandica* WP_017711629 and *Acaryochloris* RCC1774 WP_110987361 have HCAR activity. Taken together, it is shown that *P. hollandica* and *Acaryochloris* RCC1774 have the potential to complete the Chl cycle.

## Catalytic specificity of HCAR and BciB

Previous studies showed that HCAR and BciB show sequence similarity (Ito and Tanaka 2014). A phylogenetic analysis indicates that HCAR arose within the cluster of BciB during evolution. Interestingly, it was shown that cyanobacterial BciB has

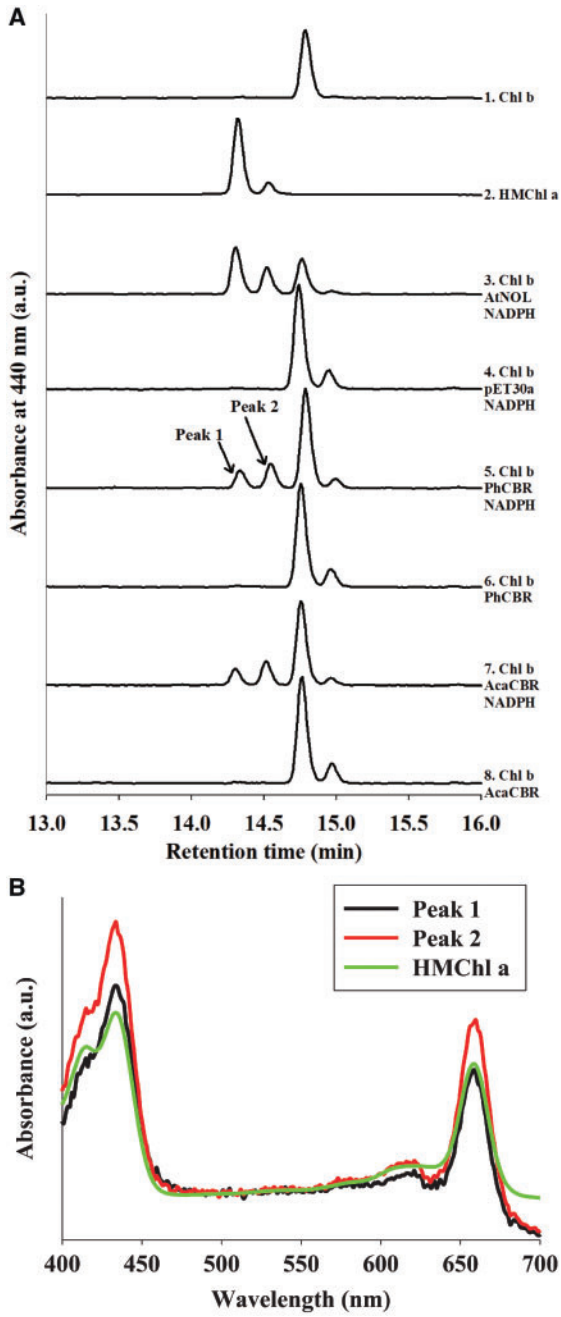


**Fig. 5** Enzymatic analysis of CBR of *P. hollandica* and *Acaryochloris* RCC1774. (A) HPLC profiles of the pigments after incubation of Chl with recombinant proteins. Chl *b* was incubated with the lysate of *E. coli* expressing PhCBR and AcaCBR with or without NADPH. Detected peaks of each pigment at 440 nm were identified by their retention time and absorption spectrum. Arabidopsis NOL was used as a positive control. 1, Chl *b*; 2, HMChl *a*; 3, lysate of *E. coli* expressing *Arabidopsis* NOL and Chl *b*, incubated with NADPH; 4, lysate of *E. coli* having pET30a and Chl *b*, incubated with NADPH; 5, lysate of *E. coli* expressing PhCBR and Chl *b*, incubated with NADPH; 6, lysate of *E. coli* expressing PhCBR and Chl *b*, incubated without NADPH; 7, lysate of *E. coli* expressing AcaCBR and Chl *b*, incubated with NADPH; 8, lysate of *E. coli* expressing AcaCBR and Chl *b*, incubated without NADPH. AtNOL, *Arabidopsis* NOL; PhCBR, *P. hollandica* CBR; AcaCBR, *Acaryochloris* RCC1774 CBR. (B) The absorption spectrum of HMChl *a*, where Peak 1 and Peak 2 were found in the reaction mixture of the lysate of *E. coli* expressing PhCBR and NADPH, as illustrated in (A).

promiscuous HCAR activity (Ito and Tanaka 2014), which might have enabled the enzyme evolution from BciB to HCAR. By contrast, green plant HCAR has no 8-vinyl reductase activity, although these two enzymes have high sequence similarity, which is hypothesized to be a result of evolutional fitting to the new substrate (HMChl *a*; Ito and Tanaka 2014). We hypothesized that promiscuous activity (such as HCAR activity in *Synechocystis* BciB) is only subjected to evolutional selection when this activity competes with the genuine activity, such as that in green algae and plants. To test this hypothesis, we examined the substrate specificity of the four gene products, *Acaryochloris* RCC1774 BciB (PZD72398), *P. hollandica* BciB (WP_044076442), *Acaryochloris* RCC1774 HCAR (WP_110987361) and *P. hollandica* HCAR (WP_017711629). As a control experiment, *Synechocystis* PCC6803 (hereafter Synechocystis) BciB (Slr1923) was used in the HCAR and BciB assays. In this study, the same volumes of the soluble fraction of *E. coli* lysate were used for the enzymatic assay. Exceptionally, the extract containing recombinant Synechocystis BciB was diluted by four times to adjust the protein levels (**Fig. 4**), because the protein level of Synechocystis BciB was much higher than those of other proteins.

We tested promiscuous HCAR activity of BciB from *P. hollandica* and *Acaryochloris* RCC1774. Instead of HMChl *a*, we used 7-hydroxymethyl chlorophyllide *a* (HMChlide *a*) for the HCAR assay of BciB because our previous experiment (Ito and Tanaka 2014) showed that Synechocystis BciB prefers this pigment to HMChl *a* in the in vitro HCAR assay. We confirmed that the recombinant BciB proteins of *P. hollandica* and *Acaryochloris* RCC1774 are able to use Chlide as a substrate for 8-vinyl group reduction (BciB) reaction (Supplementary Fig. S3). After incubation of BciB proteins of *P. hollandica* and *Acaryochloris* RCC1774 with HMChlide *a*, Chlide *a* was not detected (**Fig. 7B**, line 3 and line 4), whereas BciB of Synechocystis produced Chlide *a* after incubation (**Fig. 7B**, line 5).

Subsequently, we tested the 8-vinyl reductase (BciB) activity of HCAR recombinant proteins. After incubation of HCARs of *P. hollandica* and *Acaryochloris* RCC1774 with divinyl Chl *a*, the monovinyl form of Chl *a* was not detected (**Fig. 6B**). We noted that the absorbance spectra of minor peaks (Peak 3 and Peak 4) were similar to those of divinyl Chl *a* (**Fig. 6C**), indicating that they are most likely divinyl Chl *a*′, which might have been formed during the assay. The lack of promiscuous BciB activity in HCAR recombinant proteins was also examined using divinyl Chlide *a* as a substrate. We confirmed that these proteins are able to reduce HMChlide *a* (Supplementary Fig. S4, line 5 and line 7) in our assay conditions, suggesting that they accept Chlide as a substrate. The monovinyl form of Chlide *a* was not detected after the 8-vinyl reductase (BciB) assay (Supplementary Fig. S4, line 12 and line 13). The results show that *P. hollandica* and *Acaryochloris* RCC1774 HCARs do not have BciB activity in these assay conditions. Collectively, our results indicate that HCAR from *P. hollandica* and *Acaryochloris* RCC1774 did not show detectable promiscuous activity. These results support our evolutional fitting hypothesis mentioned above.

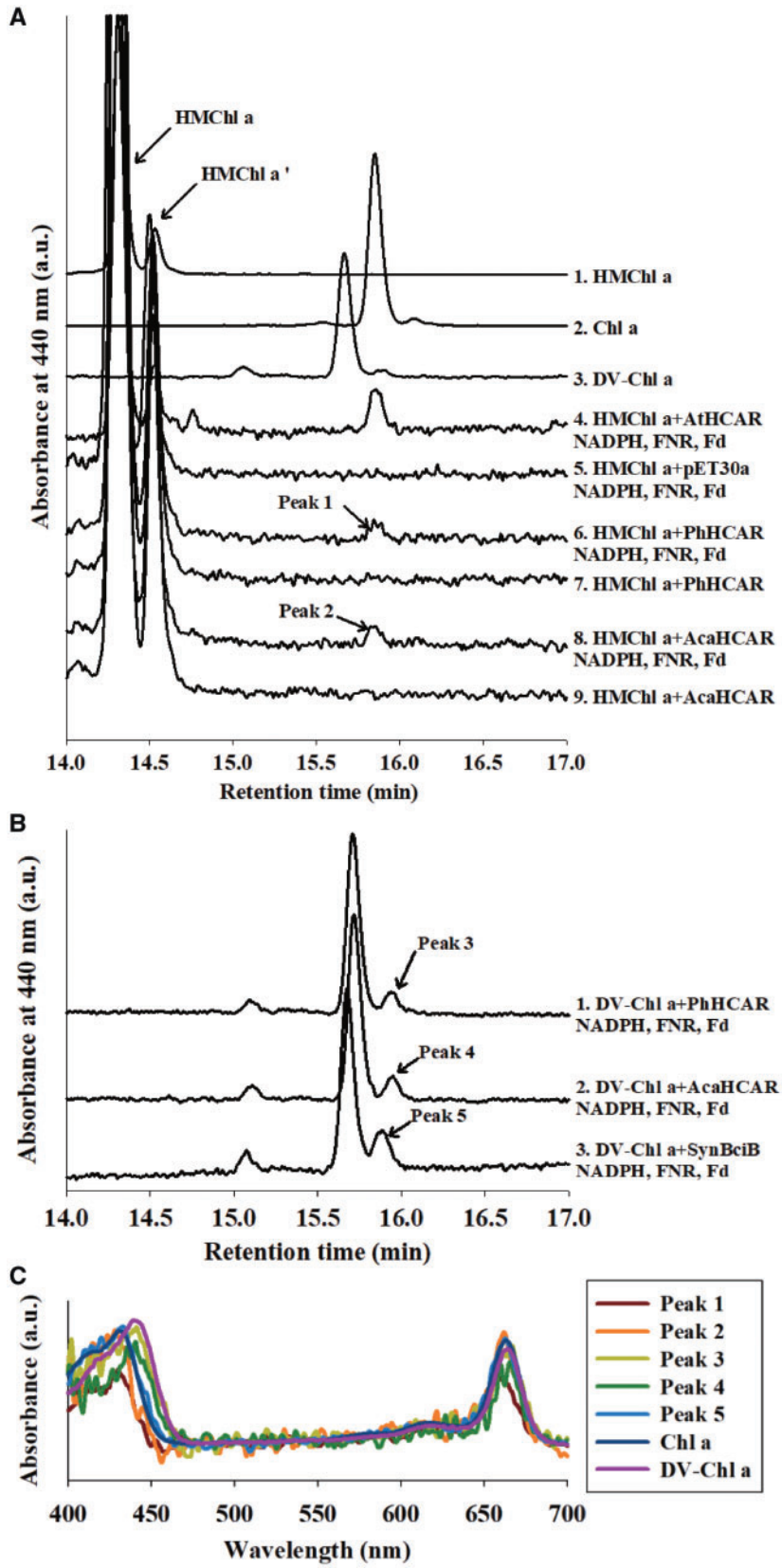


**Fig. 6** Enzymatic analysis of HCAR of *P. hollandica* and *Acaryochloris* RCC1774. (A) HPLC profiles of the pigments after incubation of Chl with recombinant proteins. HMChl *a* was incubated with the lysate of *E. coli* expressing PhHCAR and AcaHCAR with and without NADPH, FNR and Fd. Detected peaks of each pigment at 440 nm were identified by their retention time and absorption spectrum. 1, HMChl *a*; 2, Chl *a*; 3, divinyl

(continued)

## Discussion

### The Chl cycle in cyanobacteria containing Chl *b*

We showed in this report that cyanobacteria containing Chl *b*, *P. hollandica* and *Acaryochloris* RCC1774, have CAO, CBR and HCAR orthologs, and the latter two of them showed CBR and HCAR enzymatic activities, respectively (**Figs. 5, 6**). These results indicate that cyanobacteria containing Chl *b* have the potential to complete the Chl cycle. *Acaryochloris* RCC1774 and *P. hollandica* grow in shallow seas and freshwater, respectively, where light conditions change dynamically. It is likely that these two organisms need to control the antenna size in response to light conditions, as in green algae. In *P. hollandica*, Chl *b* is localized in Chl *a*/*b*-binding proteins (encoded by PCB genes) which are not related to LHC (Bumba et al. 2005, Herbstová et al. 2010). The antenna size and Chl *a*/*b* ratio are dynamically changed in response to light intensity in *P. hollandica* (Burger-Wiersma and Post 1989). However, the mechanisms of antenna size regulation are different between *P. hollandica* and green plants, because the Chl *a*/*b* ratio becomes low under high light conditions in *P. hollandica* (Burger-Wiersma and Post 1989) but high in green plants. There are no reports concerning the changes in Chl *a*/*b* ratio in *Acaryochloris* RCC1774, whereas other *Acaryochloris* species containing Chl *d* change Chl *d*/*a* ratios in response to light conditions (Duxbury et al. 2009). If a change in the Chl *a*/*b* ratio is linked to the antenna size regulation in those cyanobacteria, the Chl cycle might play a role in light acclimation.

In plants, the Chl *b*-to-*a* conversion is an important step for Chl breakdown during leaf senescence (Kusaba et al. 2007), because the Chl-degrading enzymes of plants cannot catabolize Chl *b*-type pigments that contain a formyl group at the C7 position. It is tempting to speculate that the Chl cycle could also play an important role in Chl *b* degradation in cyanobacteria containing Chl *b*. Physiological and genetic evidence will be necessary to determine if the Chl cycle functions for light acclimation and Chl degradation in these cyanobacteria.

We could not find any *HCAR* or *BciB* homologs in *Prochlorococcus*. This cyanobacterium uses divinyl Chl instead of monovinyl Chl (Barrera-Rojas et al. 2018); therefore, a lack of *BciB* homologs would be reasonable. Our analysis presented in this study indicates that *Prochlorococcus* does not have the Chl cycle either. It is reported that *Prochlorococcus* has evolved into high-light-adapted and low-light-adapted strains (Rocap et al. 2003), which have genetically adapted to different light niches. The low-light-adapted *Prochlorococcus* strain is found in deep seas. It has a large number of PCB genes and a low Chl *a*/*b* ratio. By contrast, the high-light-adapted *Prochlorococcus* strain has a high Chl *a*/*b* ratio. Therefore, there is no strong demand for an ability to change their antenna size dynamically. It is also reported that *Prochlorococcus* evolved to reduce their genome size to decrease the demand of nitrogen in oligotrophic environments (García-Fernández et al. 2004), which might also account for the absence of the Chl cycle in *Prochlorococcus*.

One potential drawback in losing the Chl cycle might be the loss of the ability to degrade Chl *b*. This is because one of the enzymes of Chl degradation pathway (pheophorbide *a* oxygenase) does not catalyze the oxidation of the substrate if the pheophorbide has a formyl group on C7 position (Hörtensteiner et al. 1995). At present, it is not clear whether *Prochlorococcus* has a novel Chl *b*-to-*a* conversion pathway or a distinct Chl breakdown pathway, which is able to catabolize Chl *b* without converting it to Chl *a*. It is also possible to speculate that cyanobacterial pheophorbide *a* oxygenase accepts Chl *b*-derived pigments, as *Auxenochlorella protothecoides* (*Chlorella protothecoides*) produces red Chl catabolites with the C7 formyl group (Iturraspe et al. 1994). Otherwise, it is also possible to assume that the bacterium simply excretes Chl *b*-type molecules. Further studies are needed to elucidate Chl degradation in *Prochlorococcus*.

*Acaryochloris* containing Chl *b* has the genes having the enzymatic activities to complete the Chl cycle by which Chl *a* and Chl *b* are interconverted. Other *Acaryochloris* produces Chl *d* instead of Chl *b* (Partensky et al. 2018). These *Acaryochloris* are expected to have the interconversion pathway of Chl *a* and Chl *d*, which would be beneficial for altering the Chl *a*/*d* ratio corresponding to the intensity of far-red light (Duxbury et al. 2009). It should be noted that *Acaryochloris marina* has both *BciA* and *BciB* and these two genes are considered to be involved in Chl biosynthesis (Chen et al. 2016). Identification of the enzymes responsible for the Chl *a* and Chl *d* interconversion is indispensable for understanding the acclimation of *Acaryochloris* containing Chl *d*.

### Evolution of the enzymes of the Chl cycle

It is suggested that the promiscuous activity of the enzymes is kept at a low level if the activity is harmful to the cells (Khersonsky and Tawfik 2010). The BciB of Synechocystis has

**Fig. 6** Continued

Chl *a*; 4, lysate of *E. coli* having Arabidopsis HCAR and HMChl *a*, incubated with NADPH, FNR and Fd; 5, lysate of *E. coli* having pET30a and HMChl *a*, incubated with NADPH, FNR and Fd; 6, lysate of *E. coli* expressing PhHCAR and HMChl *a*, incubated with NADPH, FNR and Fd; 7, lysate of *E. coli* expressing PhHCAR and HMChl *a*, incubated without reductant; 8, lysate of *E. coli* expressing AcaHCAR and HMChl *a*, incubated with NADPH, FNR, and Fd; 9, lysate of *E. coli* expressing AcaHCAR and HMChl *a*, incubated without reductant. DV-Chl a, divinyl Chl *a*. AtHCAR, *Arabidopsis* HCAR; PhHCAR, *P. hollandica* HCAR; AcaHCAR, *Acaryochloris* RCC1774 HCAR. (B) HPLC profiles of the pigments after incubation of Chl with recombinant proteins. Divinyl Chl *a* was incubated with the lysate of *E. coli* expressing PhHCAR, AcaHCAR and SynBciB with NADPH, FNR and Fd. Detected peaks of each pigment at 440 nm were identified by their retention time and absorption spectrum. 1, lysate of *E. coli* expressing PhHCAR and divinyl Chl *a*, incubated with NADPH, FNR, and Fd; 2, lysate of *E. coli* expressing AcaHCAR and divinyl Chl *a*, incubated with NADPH, FNR, and Fd; 3, lysate of *E. coli* expressing SynBciB and divinyl Chl *a*, incubated with NADPH, FNR, and Fd. SynBciB, Synechocystis BciB. (C) The absorption spectrum of Chl *a*, where Peak 1 and Peak 2 were found in the reaction mixture of the lysate of *E. coli* expressing PhHCAR and AcaHCAR with reductant, as illustrated in (A), and Peak3, Peak 4 and Peak 5 were found in the reaction mixture of the lysate of *E. coli* expressing PhHCAR, AcaHCAR and SynBciB with reductant, as illustrated in (B).

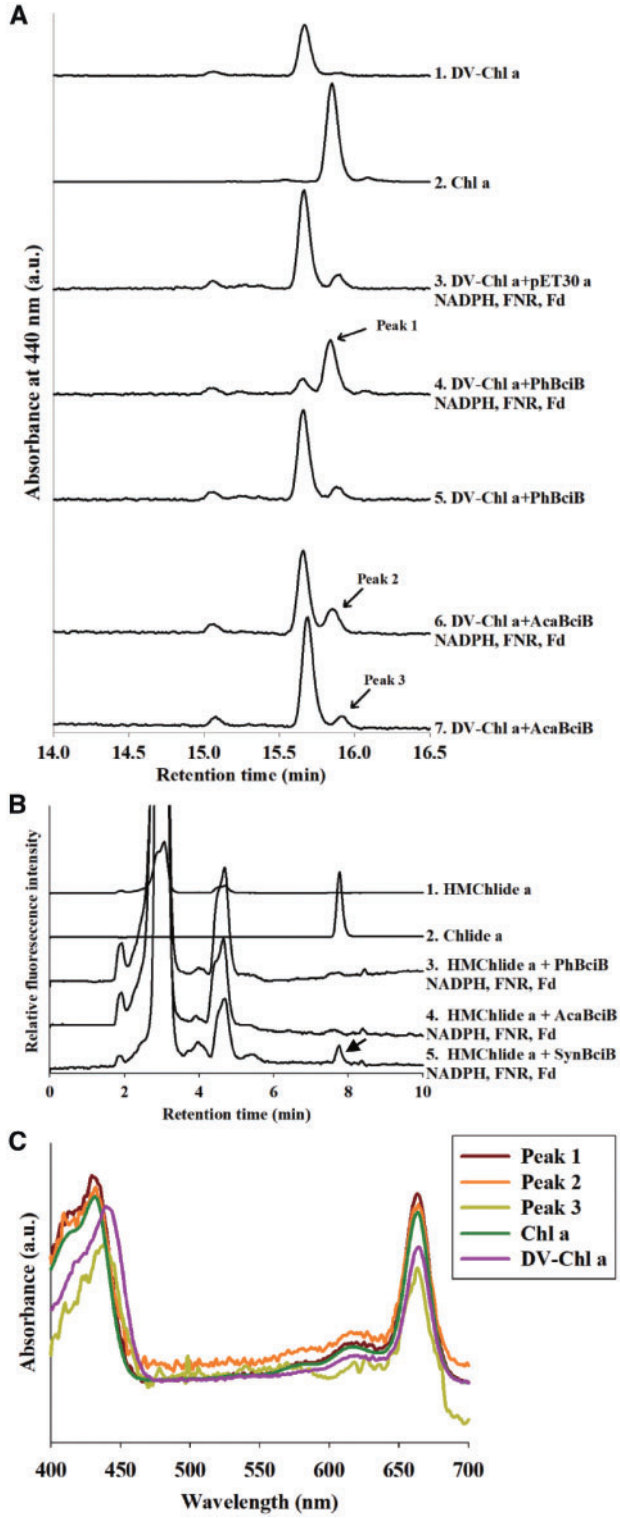


**Fig. 7** Enzymatic analysis of BciB of *P. hollandica* and *Acaryochloris* RCC1774. (A) HPLC profiles of the pigments after incubation of Chl with recombinant proteins. Divinyl Chl *a* was incubated with the lysate of *E. coli* expressing PhBciB and AcaBciB with or without NADPH, FNR and Fd. Detected peaks of each pigment at 440 nm were identified by their retention time and absorption spectrum. 1, Divinyl Chl *a*; 2, Chl *a*; 3, lysate of *E. coli* having pET30a and divinyl Chl *a*, incubated with NADPH, FNR and Fd; 4, lysate of *E. coli* expressing PhBciB and divinyl Chl *a*, incubated with NADPH, FNR and Fd; 5, lysate of *E. coli* expressing PhBciB and divinyl Chl *a*, incubated without reductant; 6. lysate of *E. coli* expressing AcaBciB and divinyl Chl *a*, incubated with NADPH, FNR and Fd; 7, lysate of *E. coli* expressing AcaBciB and divinyl Chl *a*, incubated without reductant. (B) HPLC profiles of the pigments after incubation of Chlide with recombinant proteins. HMChlide *a* was incubated with the lysate of *E. coli* expressing PhBciB, AcaBciB and SynBciB with NADPH, FNR and Fd. Detected peaks of each pigment at 680 nm fluorescence were identified by their retention time. The arrow indicates the produced Chlide *a*. 1, HMChlide *a*; 2, Chlide *a*; 3, lysate of *E. coli* expressing PhBciB and HMChlide *a*, incubated with NADPH, FNR and Fd; 4, lysate of *E. coli* expressing AcaBciB and HMChlide *a*, incubated with NADPH, FNR and Fd; 5, lysate of *E. coli* expressing SynBciB and HMChlide *a*, incubated with NADPH, FNR, and Fd. DV-Chl, divinyl Chl *a*; PhBciB, *P. hollandica* BciB; AcaBciB, *Acaryochloris* RCC1774 BciB; SynBciB, Synechocystis BciB. (C) The absorption spectrum of Chl *a*, where Peak 1, Peak 2 and Peak 3 were found in the reaction mixture of the lysate of *E. coli* expressing PhBciB and AcaBciB with or without reductant, as illustrated in (A).

high sequence similarity to green plant HCAR and the BciB has promiscuous HCAR activity (Ito and Tanaka 2014). This promiscuous activity is not harmful to cyanobacteria because they do not have the substrate (HMChl *a*) for this promiscuous activity in the cell. This broad substrate specificity and catalytic activity would contribute to the enzyme to have high catalytic activity of the primary reaction because high specificity accompanies the low catalytic activity (Khersonsky and Tawfik 2010). In this study, Synechocystis BciB showed promiscuous HCAR activities, whereas neither of *P. hollandica* and *Acaryochloris* RCC1774 BciBs showed this activity in our assay conditions. Nevertheless, the possibility cannot be excluded that these BciB homologs show HCAR under different assay conditions.

Khersonsky and Tawfik (2010) assume that if a promiscuous activity is harmful, the enzyme evolves to lose this activity. By contrast, if the promiscuous activity is not harmful, the activity will be retained (Khersonsky and Tawfik 2010). We hypothesize that the BciB of *P. hollandica* and *Acaryochloris* RCC1774 have evolved to gain higher substrate specificity and have lost their promiscuous HCAR activity, in contrast to Synechocystis BciB. We speculate that having both genuine and promiscuous HCAR activities in the same cell might interfere with the regulation of the Chl cycle in Chl *b*-containing cyanobacteria, while promiscuous HCAR activity should not have any harm in Chl *b*-less cyanobacteria. The same phenomenon is observed with HCAR of the green plants and cyanobacteria containing Chl *b*. These HCARs have no BciB activity because these organisms have another 8-vinyl reductase.

Our phylogenetic analysis (**Fig. 3B**) shows that BciB and HCAR homologs form different clades. One scenario to explain this result is that BciB was duplicated in a cyanobacterium and evolved to possess more specific substrate recognition ability of HMChl *a* in Chl *b*-containing cyanobacteria. It is possible to assume that a common ancestor of Chl *b*-containing cyanobacteria had a duplicated BciB ortholog, or that a BciB ortholog, which had HCAR activity was horizontally transferred to one of the ancestors of Chl *b*-containing cyanobacteria. HCARs of cyanobacteria and green plants form a single cluster, suggesting

that green plant HCAR evolved from cyanobacterial HCAR. Considering the similar tree topologies of CAO and HCAR sequences, it is tempting to hypothesize that HCAR might be transferred with CAO during an endosymbiotic event.

The phylogenetic tree of CBR is more complicated: CBRs do not form a single cluster (**Fig. 3A**). It is not evident from the phylogenetic tree whether green plant CBR is derived from cyanobacterial CBR or appeared independently. In either case, green plant CBR is assumed to be derived from cyanobacterial genes. In eukaryotes, red algae and brown algae have homologs of green plant CBR. The tree topology of CBR indicates that CBR homologs of red and brown algae are evolved from CBR. It is not clear at this stage whether an ancestor of those algae that do not produce Chl *b* but contain CBR homologs had synthesized Chl *b* or not. It should be noted that we also found homologs in green filamentous photosynthetic bacteria. A plausible explanation for the distribution of CBR homologs in the organisms that do not produce Chl *b* would be that those organisms obtain CBR homologs by either vertical or horizontal transfer at a certain stage of evolution, but they evolved to show other enzymatic activities than the reduction of Chl *b*. It is known that the short-chain dehydrogenase family to which CBR belongs shows remarkable diversification to catalyze various reactions (Kavanagh et al. 2008). The distribution of CBR may illustrate one example of such diversification of the short-chain dehydrogenase family.

## Materials and Methods

### Phylogenetic tree analysis

The protein sequences were obtained from the databases Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and NCBI (https://www.ncbi.nlm.nih.gov/). We used a broad variety of organisms from prokaryotes to green plants to construct a reasonable phylogenetic tree. CBR, HCAR and its homolog sequences were digested by M-Coffee and evaluated (Moretti et al. 2007). In the M-Coffee analysis, we only employed the amino acid residues which assessed the 'good' (displayed by red color) and the leftovers, which were tagged either 'BAD' or 'AVG', were clipped off from the alignment in each protein (Supplementary Tables S1, S2). The phylogenetic trees were constructed by using the maximum likelihood model and IQ-TREE version 1.6.9 (Trifinopoulos et al. 2016). Phylogenetic analysis was operated based on the 1,000 bootstrap replicants in ultrafast mode. The best-fitting amino acid substitution model was searched and employed automatically; LG+G4 was applied for both proteins.

### Strains and culture conditions

*Acaryochloris* RCC1774 was obtained from the Roscoff Culture Collection. *Acaryochloris* RCC1774 and *P. hollandica* were grown in IMK medium (Nihon Pharmaceutical, Tokyo, Japan) and BG11 medium at 23°C under a 16 h photoperiod at a light condition of 2.5 μmol photons·$m^{-2}$·$s^{-1}$ without shaking.

### Extraction and analysis of pigments

Cells were harvested by centrifuging at 20,000 × *g* for 1 min. The pellet was suspended in methanol and centrifuged at 20,000 × *g* for 10 min. The supernatant was immediately subjected to HPLC equipped with a diode array detector (SPD-M10A, Shimadzu, Kyoto, Japan) or a fluorescence detector (RF-20A, Shimadzu). The pigments were separated through a Symmetry C8 column (4.6 × 150mm, Waters, Milford, MA) with a gradient from eluent A [methanol:acetonitrile:aqueous pyridine solution (0.25 M pyridine; 50:25:25 v:v:v)] to eluent B [methanol:acetonitrile:acetone (20:60:20 v:v:v)] at a flow rate of 1 ml·$min^{-1}$ at 40°C (Shimoda et al. 2016). Detected peaks of each pigment at 440 nm were identified by their retention time and absorption spectrum. The absorption spectrum of each peak was corrected from the background absorbance.

### Cloning of CBR, HCAR and BciB from *Acaryochloris* RCC1774 and *P. hollandica*

Each coding region of CBR, HCAR and BciB derived from *Acaryochloris* RCC1774 (WP_110986784.1, PZD72038.1 and PZD72398.1) and *P. hollandica* (WP_081599361.1, WP_017711629.1 and WP_044076442.1) was amplified by polymerase chain reaction from the genomic DNA of each species. The primers used for amplification are shown in Supplementary Table S3. Amplified genes are cloned into pET-30a (+) vectors (Novagen, Madison, WI) containing a His6 tag at the C terminus using the *Nde*I and *Xho*I sites through an in-fusion cloning system (Clontech, Mountain View, CA). Synechocystis BciB (Slr1923) was prepared as reported previously (Ito et al. 2008).

### Expression and detection of recombinant proteins

The constructed plasmids for protein expression were introduced into *E. coli* (BL21). *Escherichia coli* was grown and recombinant protein was expressed in an auto-induction medium (Studier 2005) at 18°C with 130 rpm shaking. When the cell was fully saturated, 500 μl of the cell was harvested by centrifuge at 20,000 × *g* for 2 min. The pellet was resuspended with 500 μl of BugBuster Protein extraction reagents (Novagen) with 0.1% benzonase (Novagen). Immunoblotting analysis was employed to determine the expression of the recombinant proteins because expression levels were too low to be detected by Coomassie Blue Brilliant staining. After centrifugation at 20,000 × *g* for 10 min, the supernatant of the *E. coli* lysate was mixed with the same amount of sample buffer for SDS-PAGE [125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% (w/v) sucrose, 5% (v/v) 2-mercaptoehanol, and a little bit of bromophenol blue], and subjected to SDS-PAGE. For immunoblotting analysis, proteins were transferred to a polyvinylidene difluoride film. Using the antibodies for Histidine tag (Anti-His-tag mAb-HRP-DirecT, MBL, Nagoya, Japan) and Western Lighting Plus-ECL (PerkinElmer Life Science, Waltham, MA), proteins were detected by fluorescence imaging.

### Preparation of the Chl derivatives

Divinyl Chl was prepared from a Synechocystis mutant that lacks BciB (Slr1923; Ito et al. 2008). HMChl *a* was prepared through the reduction of Chl *b* using $NaBH_4$ according to a previous report (Holt 1959). Chlide derivatives were prepared by removing the phytyl chain through hydrolysis by chlorophyllase (Ito and Tanaka 2014).

### Enzyme assay

*Escherichia coli* lysate (50 μl) prepared as described above was used for the enzymatic assay. For CBR analysis, we added 1 μl of 50 mM NADPH. For HCAR and BciB analysis, we provided 1 μl of spinach ferredoxin-$NADP^+$ reductase (FNR; 0.1 mg·$ml^{-1}$, Sigma-Aldrich, St. Louis, MO), 1 μl of spinach ferredoxin (Fd; 1 mg·$ml^{-1}$, Sigma-Aldrich) and 1 μl of 50 mM NADPH to *E. coli* lysate. The pigments were solubilized with DMSO and used 0.5 μl of the solution, which contains 500 pmol of pigments. The mixture was incubated at 37°C for 1 h, and the reaction was stopped by adding 200 μl of acetone. After centrifugation at 20,000 × *g* for 10 min, the supernatant was analyzed by HPLC.

## Supplementary data

Supplementary data are available at PCP online.

## Funding

JSPS, KAKENHI [15H04381 to A.T.; 16H06554 and 17K07431 to R.T.; and 17K07430 to H.I.].

## Acknowledgments

We thank Dr. Atsushi Takabayashi (Hokkaido University) for assistance with phylogenetic analysis.

## Disclosures

The authors have no conflicts of interest to declare.

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