博士学位論文

The presence of the chlorophyll cycle in chlorophyll b-containing cyanobacteria implicated by the in vitro activity assay

（In vitro における活性測定によって示された、クロロフィル b を合成するシアノバクテリアにおけるクロロフィルサイクルの存在）

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The presence of the chlorophyll cycle in chlorophyll b-containing cyanobacteria implicated by the in vitro activity assay

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Abstract

In plants, chlorophyll *a* and *b* are interconvertible by the action of three enzymes – chlorophyllide *a* oxygenase, chlorophyll *b* reductase (CBR), and 7-hydroxymethyl chlorophyll *a* reductase (HCAR). These reactions are collectively referred to as the chlorophyll cycle. In plants, this cyclic pathway ubiquitously exists and plays essential roles in acclimation to different light conditions at various developmental stages. In contrast, only a limited number of cyanobacteria species produce chlorophyll *b,* and these include *Prochlorococcus*, *Prochloron*, *Prochlorothrix*, and *Acaryochloris*. In this study, we investigated a possible existence of the chlorophyll cycle in chlorophyll-*b* synthesizing cyanobacteria. First, we selected CBR and HCAR homologues from *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 genomes and tested whether their gene products show CBR or HCAR activity *in vitro* by overexpressing them in *Escherichia coli*. All of these proteins show CBR and HCAR activity *in vitro,* respectively, indicating that both cyanobacteria possess the chlorophyll cycle. It is also found that CBR and HCAR homologues exist only in the chlorophyll *b*-containing cyanobacteria that habitat shallow seas or fresh water, where light conditions change dynamically, while they are not found in *Prochlorococcus* species that usually habitat environments with fixed lighting. Thus, it is hypothesized the chlorophyll cycle also contributes to light acclimation in cyanobacteria.

Keywords: Chlorophyll cycle, 7-hydroxymethyl chlorophyll *a* reductase (HCAR), chlorophyll *b* reductase (CBR), *Prochlorothrix hollandica*, *Acaryochloris* RCC1774

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1 Introduction

1.1 Global warming and the reason

Recently, the climate changed drastically, and this phenomenon accompanied a huge impact to human life. Moreover, people already felt these changes such as the increment of heavy rain (Japan Meteorological Agency 2015), the frequency of typhoon (Vecchi and Knutson 2008) and hardness drought (Diffenbaugh et al. 2015). In addition, humanity got tremendous damages by this phenomenon.

There are some arguments of the main reason for the climate change. Lots of people didn't believe that carbon dioxide is main reason of the change, and they insist that the force to recovery from the little ice age by few climate skeptic bloggers based on a paper (Miller et al. 2012). They insisted like that the rise of the surface temperature of earth during last few decades if the main reason of climate change and this is the natural pattern for recovery of the little ice age. However, in 2018, a report (USGCRP 2018) from the US Global Change Research Program made the period of these arguments. The report states that the main reason of global warming is an increase in the carbon dioxide concentrations in atmosphere resulted from the human activity.

1.2 The emission source of carbon dioxide and the renewable energy

Based on the report (IPCC 2007) from Intergovernmental Panel on Climate Change mentioned the major factor of greenhouse gases is carbon dioxide which got a 77% portion of temperature rise. Electricity supply by using fossil fuel dominated over 40% of the global energy related emissions of carbon dioxide. (Chisti 2013).

Even though, the reserve of petroleum base energy sources is increased by sail gas and finding new reserve under the ground, but there is no argument that these reserves has a limitation and that will be deplete. Based on this there are intensive arguments searching for the of alternative fuel that replaces fossil ones (Posten and Schaub 2009).

Unfortunately, most of the renewable energies, such as solar, wind or hydroelectricity, are not helpful to decrease the carbon dioxide, which is already in atmosphere. Due to this aspect, bioenergy which can produce through the conversion of carbon dioxide is considered to be one of the key solutions of global warming (Gerdien Meijerink (LEI), Wolter Elbersen (A&F) 2004).

1.3 Bioconversion carbon dioxide and bioenergy

Carbon Capture and Storage (CCS) is known as interception of carbon dioxide to atmosphere and store in underground or sea (Möllersten, Yan, and Moreira 2003). Bioconversion, however, is a partially different concept of CCS, because of that this technology is focused on utilizing carbon dioxide in atmosphere and making the energy source through the living organisms, such as plants and microalgae(Azar et al. 2010).

In the bioconversion technology, there are profits compared with other CCS technologies. Firstly, during the CCS process, the highest cost and energy required in the carbon capturing process, but bioconversion doesn't require this process (Stuart Haszeldine 2009). Secondly, this do not make the pipe line and use the other transport method to move carbon dioxide (Boot-Handford et al. 2014).

1.4 Biofuel

Biofuel is classified into several generations by the material and source in order to produce a fuel. The first generation of biofuel was started from the crops, such as sugar cane or corn, and this situation can be triggering a tremendous side effect to worldwide food chain and supply (European Academies Science Advisory Council 2012). Due to the this aspect, there are some doubts that it can be replace the fossil fuel and stable supply to society (International Energy Agency 2006; Moore 2008).

The second generation is utilizing lignocellulosic, for example wood, straw and waste (Sims et al. 2010). This has several strong points compared with first generation technology, which can avoid the shock to the food market and the source of the fuel from the low-quality land (Inderwildi and King 2009). However, a weak point of this process is that during the process it need to high temperature and pressure decomposition and multiple expensive catalytic synthesis steps (Moore 2008).

For the solution for overcoming these short comings, researchers proposed the third and fourth generation of biofuel which utilize the algal or designated micro creature (Lü, Sheahan, and Fu 2011). Both generations are not only using a microalga which can produce the lipid for bioenergy, but also used the biomass as a food or animal feed. In addition, some of species can produce the high value products, such as antioxidants and antibiotics (Yadav and Sen 2018).

1.5 Microalga

In this thesis, the definition of the microalgae are all types of unicellular organisms that perform photosynthesis, including prokaryotic microalgae such as cyanobacteria and eukaryotic microalgae like green algae and diatoms (Figure 1).

As I mentioned in the previous chapter, researchers studied microalgae as a potential source of biofuel because of their several advantages. Firstly, microalgae can produce 10~20 times higher amounts of oil compared with other corps or plants in the same space (Chisti 2007; Mata, Martins, and Caetano 2010). Secondly, they don't compete a land with corps because they can grow the brackish or foul water on useless land, and this is the better point compared with first generation biofuel (Haldar et al. 2018). Thirdly, almost all of microalgae require aqueous condition for growth, however, compared with crops, they consume less amount of water (Se-Kwon, Kim., Choul-Gyun 2015). Fourthly, they generally have a higher level of carbon fixation ability, which trait is favorable in terms of reducing green-house gasses (Chisti 2013).

On the other hand, there are some bottleneck to industrialization. The most critical point is that this technology is still needed to increase the productivity to reach the reasonable price for the fuel market (Pienkos and Darzins 2009; Stephens et al. 2010). In order to overcome this hurdle, researchers struggle to find an answer. For example, for saving the cost some researchers use waste water as a medium (Chinnasamy et al. 2010; Posadas et al. 2017). But, unfortunately, this approach also didn’t serve as enough solution for cost reduction in fuel production. Many researchers assume that

genetic modification may contribute to the reduction of the product cost or to the productivity of algal cells (Radakovits et al. 2010).

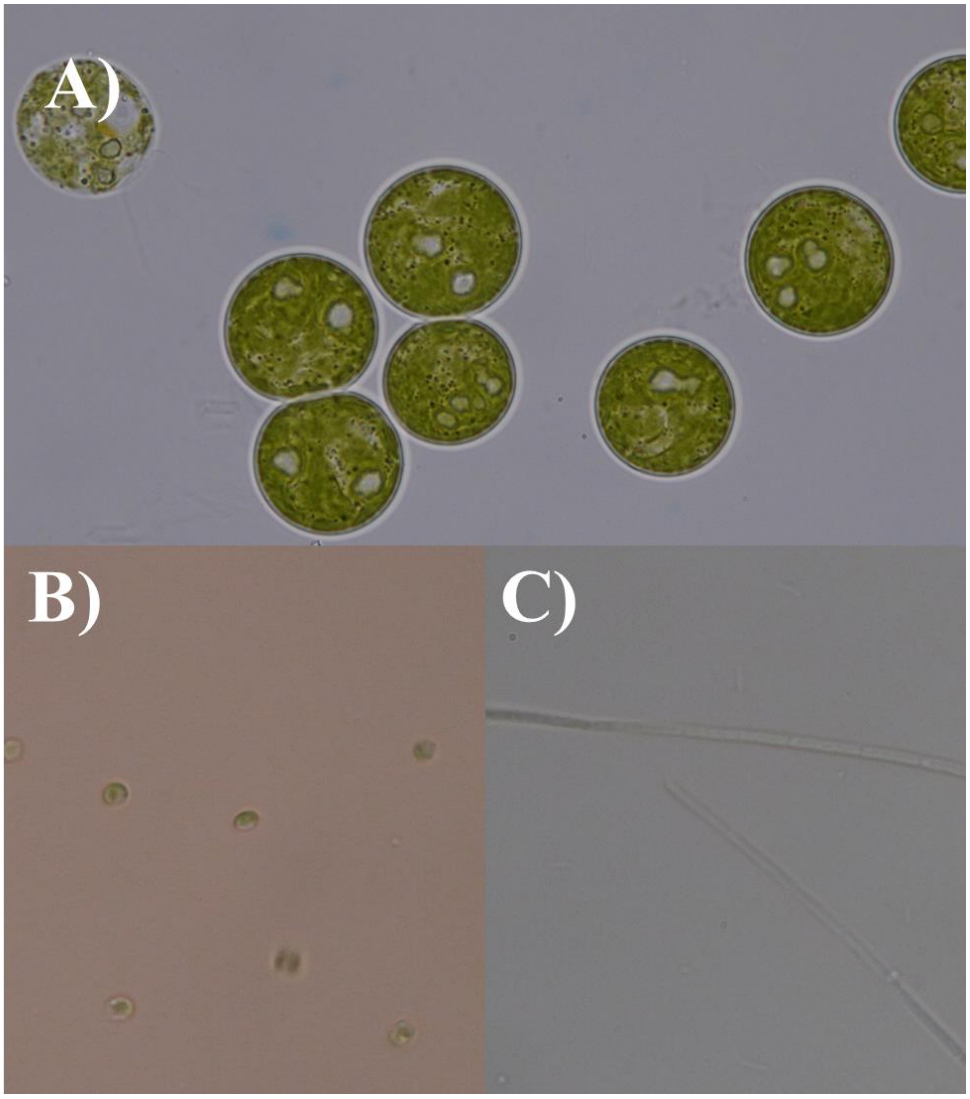

Figure 1. Cell images of various microalgae and cyanobacteria.

A) *Haematococcus pluvialis*, B) *Nannochloropsis oceanica,* C) *Prochlorothrix hollandica.*

1.6 Cyanobacteria

Cyanobacteria, normally called blue green algae, are only prokaryotes that perform oxygenic photosynthesis. (Binder 1982).

Cyanobacteria have been in the spotlight because they serve as not only a key for seeking a knowledge for chloroplast evolution in higher plants (Gray and Doolittle 1982), but also, recently, considered as potential source of source of biomass and biofuel, although this species contain the lipid below the 20% of wet mass (Harun, Danquah, and Forde 2010; John et al. 2011; Markou and Georgakakis 2011; Sialve, Bernet, and Bernard 2009). In addition, it has the 6~12 time higher energy conversion rate than terrestrial plants (Brenner 2006; Dismukes et al. 2008).

Moreover, cyanobacteria usually have short life cycles, and it is easier to make a mutant compared with microalgae such as green algae (Van Alphen et al. 2018; Donnan, L., Carvill, E. P., Gilliland, T. J., & John 1985). Recent progresses in the understanding of the biochemical pathways made it possible to build up complex genetic engineering of photosynthesis-related reactions, such as controlling the antenna size of light harvesting complex or controlling the carbon partitioning for final products (Ducat, Way, and Silver 2011; Mussgnug et al. 2007; Sanz et al. 2015; Shabestary et al. 2018).

1.7 *Prochlorothrix hollandica* and *Acaryochloris* RCC1774

In this thesis, we used the two cyanobacteria *Prochlorothrix hollandica* and *Acaryochloris* RCC1774. Normally typical cyanobacteria are not possessed the chlorophyll b which is famous as one of the parts of light harvesting complex. However, these two species contain the chlorophyll b (Herbstová et al. 2010; Partensky et al. 2018) and these types of cyanobacteria called 'green oxyphotobacteria' (Partensky and Garczarek 2011).

Prochlorothrix hollandica was isolated from the lake in Netherland at 1984 (Burger-Wiersma and Post 2008). Interesting point is that this species is paucity of the phycobilisomes, but it contain the light harvesting structure, like higher plant and microalgae, which is possessed chlorophyll a and b (Partensky and Garczarek 2011).

Acaryochloris RCC1774 is isolated by Jean-Claude Thomas (Partensky et al. 2018) and collected from the Britany coast in France (Roscoff Culture). This species is one of the members of *Acaryochloris* genus, in addition, has very specific even though this contain the both chlorophyll a and b, it also contains the phycocyanin (PC). this situation is atypical case because other green oxyphotobacteria possess almost undetectable amount, but this species contains high amount of PC (Partensky et al. 2018).

1.8 Oxygenic Photosynthesis

Generally, we are understanding the photosynthesis is the process to make a chemical compound using the sun light, carbon dioxide and water. In this process, there are two types of photosynthesis; oxygenic and anoxygenic photosynthesis. Oxygenic photosynthesis produces oxygen and uses water as an electron donor. Oxygenic photosynthesis contain two different stage; one is called light-dependent step which is energy transduction process, the other is called light-independent procedure which is carbon-fixation step (Cowgill and Redding 2012). In light-dependent procedure, the first step is light harvesting process, and this is operated by several pigments, which are called chlorophylls and carotenoids (Peter and Thornber 1991).

1.9 Chlorophyll

Chlorophylls (Chls) play a vital role in photosynthesis by harvesting the light energy and driving the electron transfer (Renger and Schlodder 2010). The comprise light-harvesting antenna systems around each photosystem (Soll and Schleiff 2004). They transfer the absorbed light energy by resonance energy transfer to the special pair of each photosystem which drives charge separation.

There are five different types of Chls in nature, which are Chl *a, b, c, d* and *f.* They show different absorbance spectra due to small changes in the side chains of chlorophyll. The common and main structure of chlorophyll is a closed tetrapyrrolic macrocycle (Francis 2000).

To achieve these photosystem restructurings, the synthesis and degradation of chlorophyll must be finely regulated in response to light environments (Masuda, Tanaka, and Melis 2003; Tanaka et al. 2002). 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 (M. 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. In 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).

1.10 Adaptation to various light conditions

Photosynthetic organisms adapted or acclimate to a wide range of light environments by using different sets of antenna systems. For example, the model green alga, *Chlamydomonas* contains chlorophyll b only in the peripheral antenna complexes, whereas other types of green algae living in deep sea have chlorophyll b in both the core and peripheral antenna complexes. Green plants alter their antenna sizes by changing the chlorophyll a/b ratio (Bailey et al. 2001). When the plants grow under low-light conditions, plants have a low chlorophyll a/b ratio and large antenna size. Therefore, the chlorophyll a/b ratio must be regulated in response to changes in the light environment.

In the biofuel industry, researchers are trying to control the phenotype of algae to ameliorate the efficiency of the photosynthesis (Peers 2014; Stephenson et al. 2011). The best photosynthetic efficiency in theory is only 8~10% of photosynthetic effectivity under light-limiting conditions. Under natural environments such as in an open-pond cultivating system of microalgae, this level of efficiency is even difficult to achieve because of the fluctuation and strong light intensity over the capacity of photosynthesis (Hambourger et al. 2009; Melis 2009). Some researchers hypothesized that the regulation of the light harvesting pigments might circumvent this problem. Then, they decreased the chlorophyll amount in light harvesting complex by genetic modification and attempted to increase the penetrated light for reaching the sunlight in the deep side of the cell cultivation system (Melis 2009).

1.11 Chlorophyll b

The majority of chlorophyll b molecules, if not all, in the chloroplast are bound to light-harvesting chlorophyll a/b-protein complexes (LHC) in chloroplasts. More than a dozen types of LHC have been identified in photosynthetic eukaryotes. One LHC typically binds about 12 chlorophylls and a few carotenoids. It is considered that no "free" chlorophyll b present in chloroplasts. Mutant analysis with *Arabidopsis* (Horie et al. 2009) and rice (Kusaba et al. 2007) demonstrated that LHC is not degraded in the chlorophyll b reductase (CBR) mutant during senescence. In vitro experiments indicated that chlorophyll b in LHC can be a substrate of recombinant CBR (Horie et al. 2009). These experiments indicate that chlorophyll b in LHC is the primary substrate of CBR in chloroplasts. CBR might have evolved to be able to catalyze chlorophyll b in LHC.

Chlorophyll b is found not only in eukaryotic green plants but also in some cyanobacterial lineages, such as *Prochlorococcus*, *Prochloron*, and *Prochlorothrix* (Palenik and Haselkorn 1992). Recently, a novel species of *Acaryochloris* was found to have chlorophyll b instead of chlorophyll d (Partensky et al. 2018). These organisms containing chlorophyll b do not form a single cluster in the phylogenetic tree but appear independently in the cyanobacterial radiation (Partensky et al. 2018). Cyanobacteria do not have light-harvesting complexes (LHC). Instead, chlorophyll b is incorporated into the prochlorophyte chlorophyll b-binding protein (PCB) (Bibby et al. 2003; Bumba, Prasil, and Vacha 2005), which is not phylogenetically related to

LHC (Roche et al. 2002). The localization of chlorophyll b in *Acaryochloris* has not been reported, but it might be present in PCB, as in *Prochlorococcus* and *Prochlorothrix*.

1.12 Regulatory mechanism of the chlorophyll contents in higher plants.

Green plants employ various strategies to regulate chlorophyll a/b ratios. Chlorophyllide *a* oxygenase (CAO) is responsible for chlorophyll b synthesis. CAO protein levels are finely regulated at the transcriptional level and protein degradation rates (Nakagawara et al. 2007; Tanaka and Tanaka 2005; Yamasato 2005). Another important reaction for regulating the chlorophyll a/b ratio is the conversion of chlorophyll b to chlorophyll a (Tanaka and Tanaka 2007). In this pathway, chlorophyll b is converted to 7-hydroxymethyl chlorophyll a by chlorophyll b reductase (CBR) (Kusaba et al. 2007), followed by the reduction to chlorophyll a by 7-hydroxymethyl chlorophyll a reductase (HCAR) (Figure 2) (Meguro et al. 2011). This pathway is responsible not only for the regulation of chlorophyll a/b ratios, but also for the degradation of chlorophyll b during senescence, because chlorophyll b must be converted to chlorophyll a before degradation (Hörtensteiner, S., Vicentini, F., & Matile 1995). Therefore, interconversion of chlorophyll a and chlorophyll b (referred to as the chlorophyll cycle) plays a crucial role in various developmental stages in green plants.

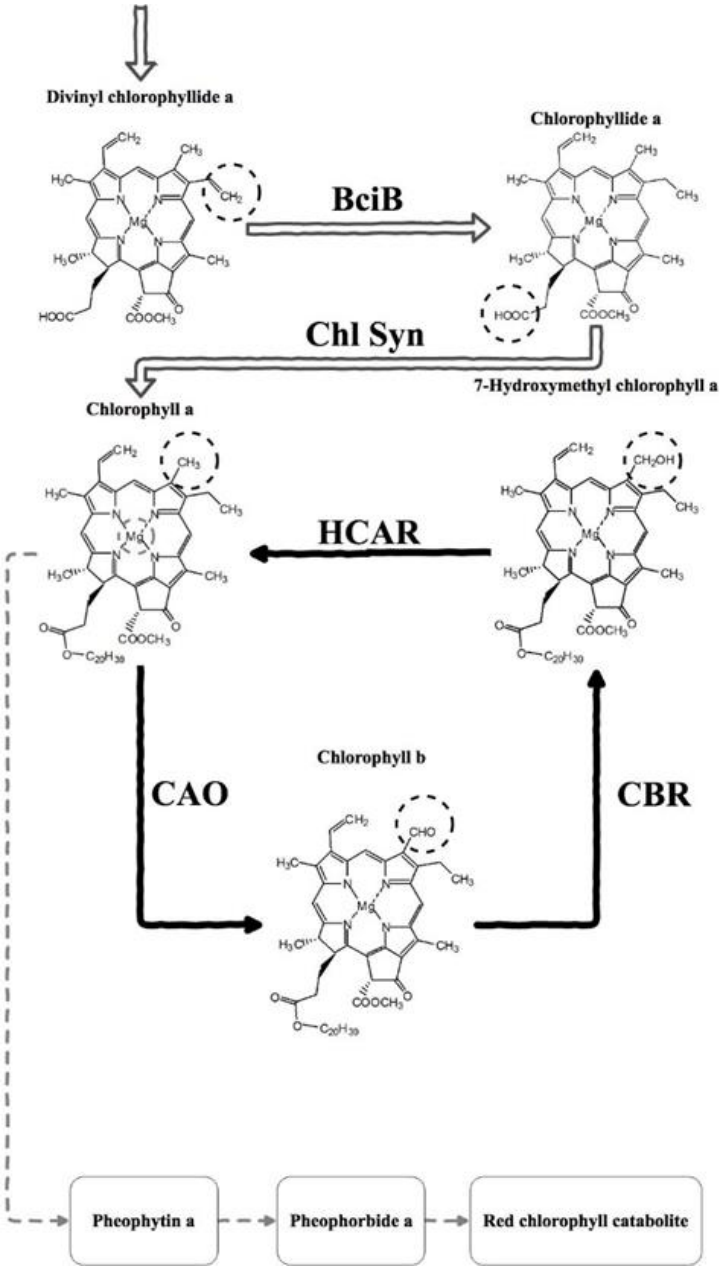

Figure 2. Chlorophyll metabolic pathway.

Outline arrow, black arrow, and dash arrow represent chlorophyll synthesis pathway, chlorophyll cycle, and chlorophyll degradation pathway, respectively. Dashed circle indicates the reaction site. Chl Syn, chlorophyll synthase.

1.13 Chlorophyll b reductase

chlorophyll b reductase simply called CBR and considers belonging to the short-chain dehydrogenase superfamily. In addition, This enzyme is working on the first step of the process of Chl b to Chl a. there are a report that this enzyme is enhance to express during the dark induce senescence (Scheumann, Schoch, and Rüdiger 1999). In higher plant, during the leaf senescence, all of the enzymes for Chl-degradation are existed in the envelop membrane in chloroplast (Matile and Schellenberg 1996), however, this enzyme localized in the inside of the thylakoid membrane (Scheumann, Schoch, and Rüdiger 1999). There are isozymes in higher plant, for example NYC1 and NOL *Arabidopsis thaliana*, but there is no report about cyanobacteria's chlorophyll b reductase.

1.14 7-hydroxymethyl chlorophyll a reductase

7-hydroxymethyl chlorophyll a reductase is simply called HCAR and this enzyme have highly similar sequence to divinyl chlorophyll vinyl reductase (DVR) (Meguro et al. 2011). The enzyme is working on the second step of the chlorophyll cycle using the HMChl a to Chl a as a substrate. Interestingly, in cyanobacteria such as *Synechocystis*, this DVR show the capacity of HCAR, although two enzyme has different substrate specificity in higher plants (Meguro et al. 2011). During the lead senescence, this enzyme take the important role in process of chlorophyll breakdown (Sakuraba et al. 2013), and normally expression of this enzyme is suppressed during dark-induced senescence (Meguro et al. 2011).

1.15 Regulation process of chlorophyll in cyanobacteria.

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

In this study, I constructed phylogenetic trees of CBR, HCAR, and their homologous genes with their homologous genes, and determined the candidates of these genes in *Prochlorothrix hollandica* and *Acaryochloris* RCC1774. Then I tried to determine the enzymatic activities using the recombinant proteins of these candidate genes and found the CBR and HCAR activities, indicating the presence of the chlorophyll cycle in cyanobacteria containing chlorophyll b.

2 Results

2.1 Microscopic image of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774

During the whole of the experiments we used two cyanobacterial species, *Prochlorothrix hollandica* and *Acaryochloris* sp. RCC1774, which contain Chl a and b. Hereafter, they are referred to as *Prochlorothrix* and *Acaryochloris*, respectively.

Acaryochloris cells are coccoid during the growth phase (Partensky et al. 2018).

Under the microscope, the size of *Prochlorothrix hollandica* got some variations, which is probably depending on their growth stages according to the previous report (Burger-Wiersma, Stal, and Mur 1989). Under light microscope, both species show green color, and under ultraviolet illumination they show red fluorescence, demonstrating the presence of chlorophyll.

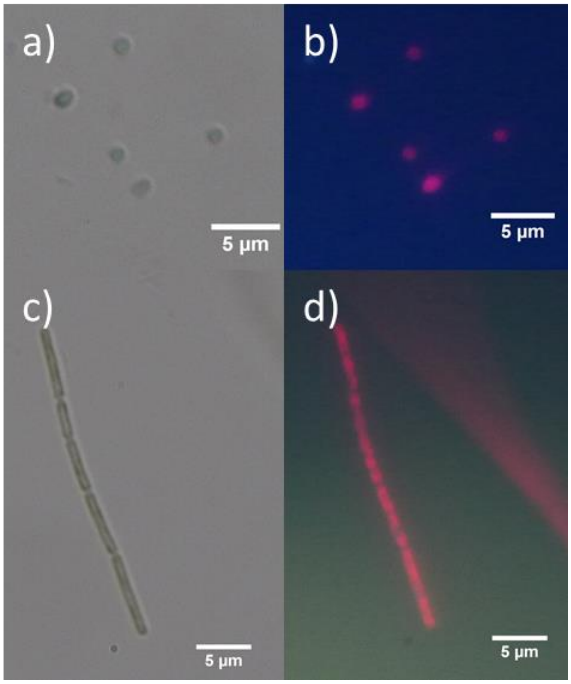

Figure 3. Cell images of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774.

a and b *Acaryochloris* RCC1774; c and d *Prochlorothrix hollandica*. b and d are showed the chlorophyll signal under the UV lamp.

2.2 Fluorescence spectra of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774

Firstly, we checked if the strains we used in this study produce Chl b in our growth conditions by monitoring their fluorescence spectra in comparison to *Synechocystis* sp. PCC6803, which does not produce Chl b at all. Figure 4 shows the excitation spectra between 400 and 600 nm with the fluorescence monitored at 684 nm. In most cases, PSII give fluorescence between 680-690 nm, thus the excitation spectra provide an estimate which wavelength of light is better absorbed and transferred to PSII. Synechocystis gives weaker fluorescence between 400 and 450 nm indicating that phycobilisomes play dominant roles in the absorption of light energy. In contrast, *Prochlorothrix* and *Acaryochloris* show stronger fluorescence when blue light (400-450 nm) is absorbed indicating that chlorophyll plays an important role light absorption. Moreover, these organisms show fluorescence excitation maxima at around 465 nm indicating that Chl b also contributes to light absorption.

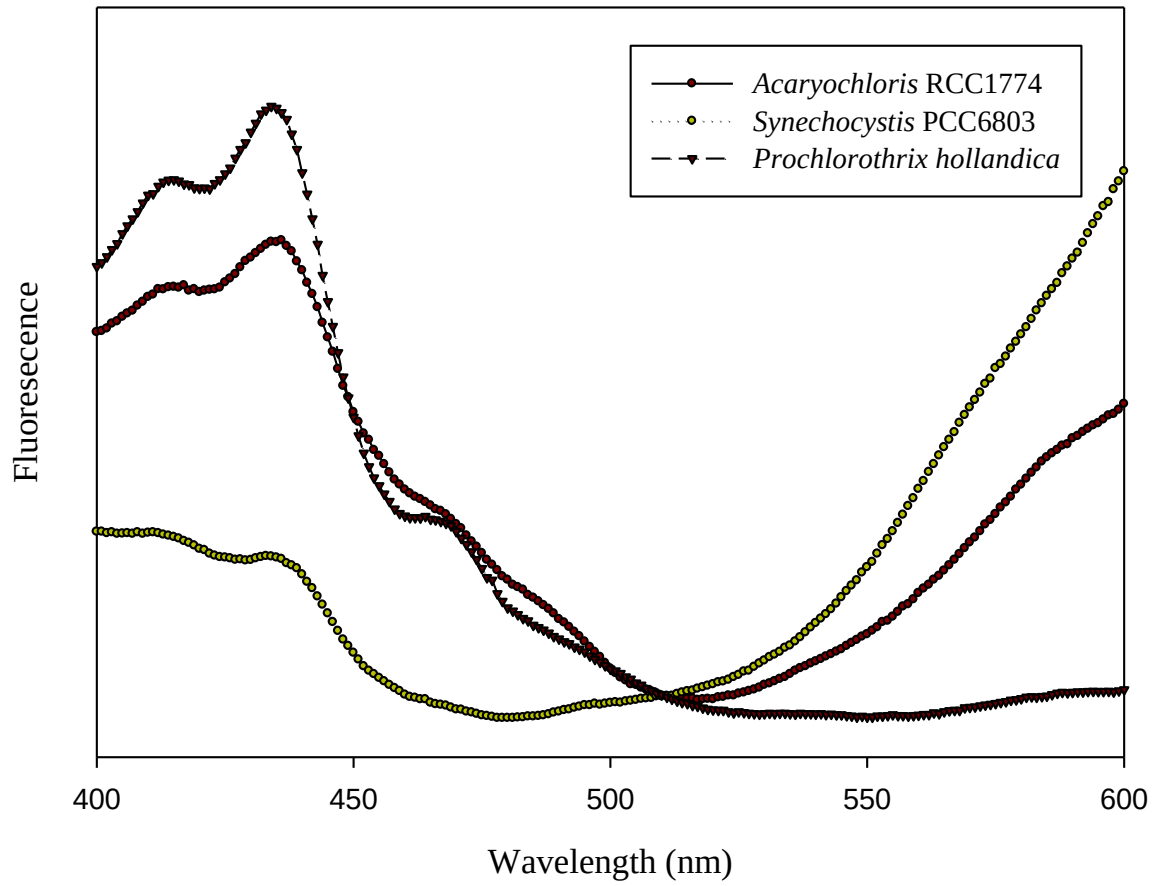

Figure 4. Excitation spectroscopic analysis of cyanobacterial cells.

(Room temperature, 684 nm fluorescence monitored)

2.3 Pigment composition of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774

Photosynthetic pigments of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 were examined by high-performance liquid chromatography (HPLC) (Figure 5). Pigment compositions were similar to those in previous reports (Partensky et al. 2018; Takaichi et al. 2012). Elution profiles of HPLC were similar between the two organisms with some differences. *Acaryochloris* RCC1774 and *Prochlorothrix hollandica* have α- and β-carotene, respectively. Both of them have ε,ε-carotene (Figure 6). Chlorophyll a/b ratio of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 were 13.5 and 6.8, respectively. *Acaryochloris* RCC1774 has divinyl protochlorophyllide a, which is a precursor of chlorophyll biosynthesis, while *Prochlorothrix hollandica* does not have this pigment. The absorbance spectrum of this pigment is similar to that of chlorophyll c, therefore, it is possible to hypothesize that it functions for light harvesting in *Acaryochloris* RCC1774 like it does in Prochloron (Larkum et al. 2006).

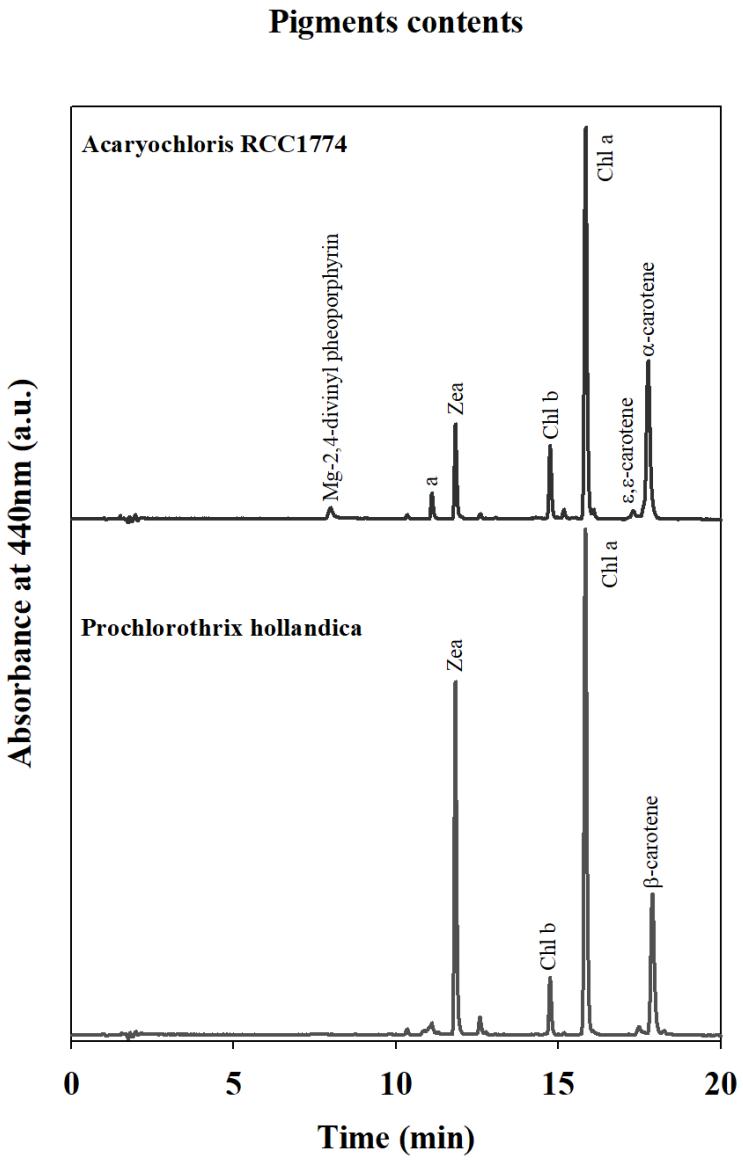

Figure 5. Pigment compositions of *Acaryochloris* RCC1774 and *Prochlorothrix hollandica*.

HPLC profiles of the pigment monitored at 440 nm. The peaks were identified by their retention time and spectrum. a, possible calolaxanthin (monohydroxy-zeaxanthin); Zea, Zeaxanthin; Chl b, chlorophyll b; Chl a, chlorophyll a.

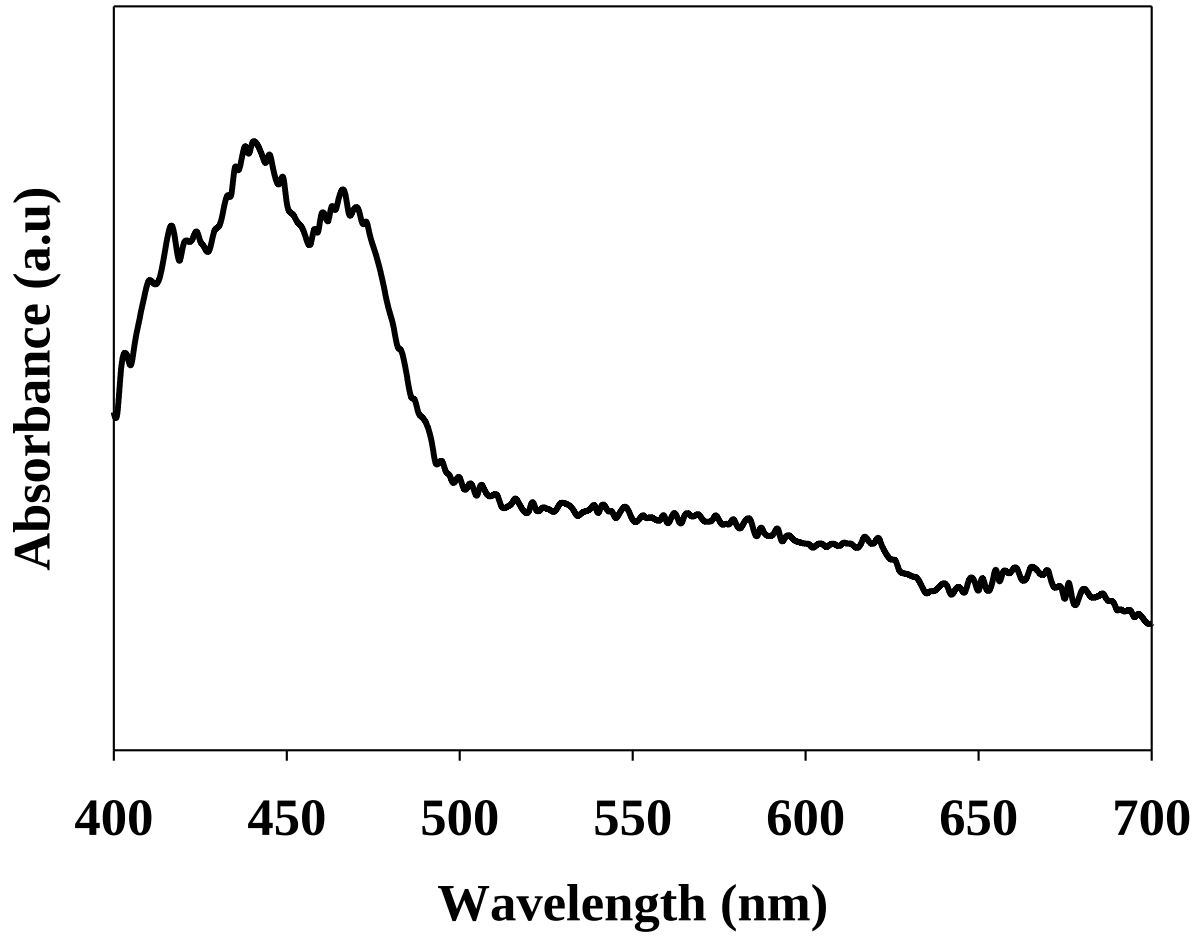

Figure 6. The absorption spectrum of ε,ε-carotene detected in *Acaryochloris* RCC1774.

2.4 Phylogenetic analysis of the chlorophyll cycle enzymes

CAO catalyzes the oxidation of a methyl group on chlorophyll a to a formyl group (Oster et al. 2000), and it is responsible for the formation of chlorophyll b in all organisms containing chlorophyll b (Tomitani et al. 1999). The phylogenetic tree of CAO (Figure 7) demonstrates the phylogenetic relationship of these organisms, indicating that green plants obtained their CAO genes from cyanobacteria.

CBR catalyzes the reduction of a formyl group on chlorophyll b to a hydroxymethyl group, which is the first step of chlorophyll b to chlorophyll 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 homologues are widely distributed not only in green plants but also in other organisms, including red algae, diatoms, cyanobacteria, and photosynthetic bacteria (Figure 8). Green plants have two CBRs, Non-Yellow Coloring 1 (NYC1) and NYC1-Like (NOL). CBR homologues of red algae and diatoms are most closely related to green plant CBRs phylogenetically, although they do not produce chlorophyll b. Interestingly, green sulfur bacteria also have CBR homologues. Genes highly homologous to CBR were found only in photosynthetic organisms, suggesting that homologous genes are related to photosynthesis. CBR homologues of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 were most distantly related to green plant CBRs phylogenetically in this tree, although these two cyanobacteria have chlorophyll b. We were not able to

conclude whether CBR homologues of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 encode active CBR enzymes because CBR homologues of these two cyanobacteria do not form a cluster with CBRs but with other cyanobacteria that do not have chlorophyll b.

In the chlorophyll cycle, 7-hydroxymethyl chlorophyll a is converted to chlorophyll a by HCAR. We retrieved its homologous genes using *Arabidopsis* HCAR as a query. Many homologous proteins were found from photosynthetic eukaryotes and cyanobacteria. The phylogenetic tree (Figure 9) 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 chlorophyll b nor HCAR. However, cyanobacteria containing chlorophyll b, *Prochlorothrix hollandica* and *Acaryochloris* RCC1774, have two homologous genes, one of which belongs to the HCAR cluster and the other to the BciB cluster. This phylogenetic analysis implicates that *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 have HCAR.

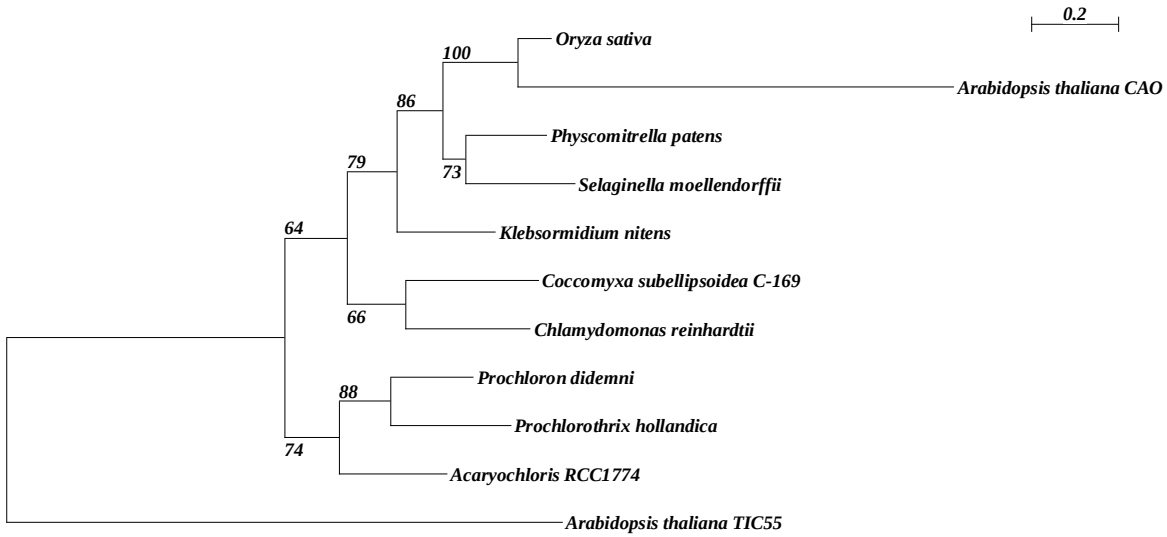

Figure 7. Phylogenetic trees of CAO.

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. *Arabidopsis thaliana* TIC55 (AT2G24820.1); *Oryza sativa* (LOC_Os10g41780.1); *Chlamydomonas reinhardtii* (AAX54904.1); *Coccomyxa subellipsoidea* C-169 (XP_005644725.1); *Acaryochloris* RCC1774 (WP_110987895.1); *Prochlorothrix hollandica* (P_017713323.1); *Arabidopsis thaliana* (AT1G44446); *Prochloron didemni* (BAA82483.1); *Klebsormidium nitens* (GAQ77793.1); *Physcomitrella patens* (XP_024360531.1); *Selaginella moellendorffi* (XP_002990495.2); *Micromonas commoda* (XP_002499624.1); *Ostreococcus lucimarinus* CCE9901 (XP_001418699.1).

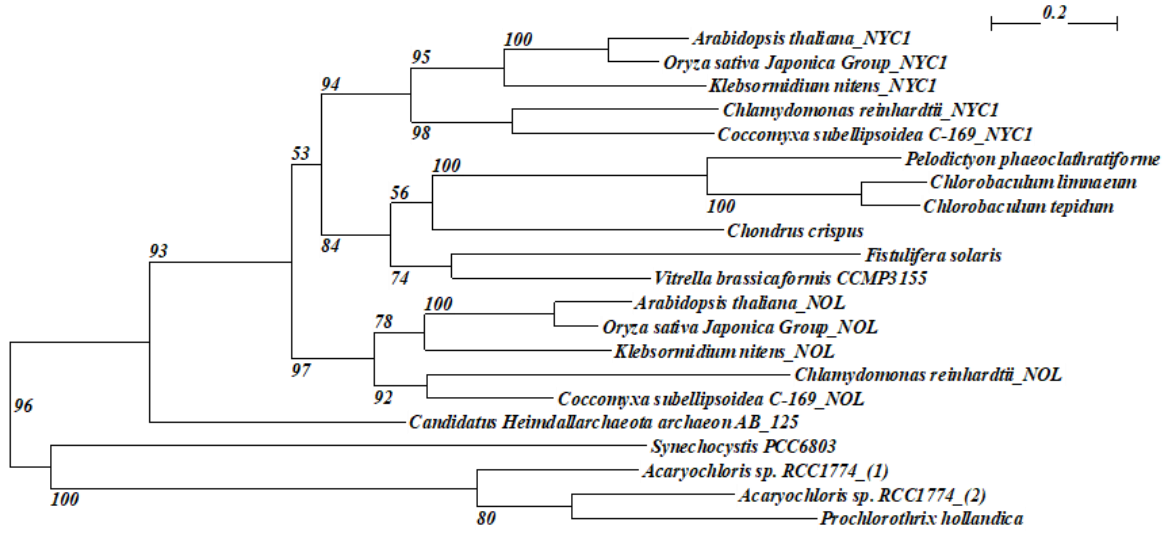

Figure 8. Phylogenetic tree of CBR.

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. *Arabidopsis* NYC1 (AT4G13250); *Arabidopsis* NOL (AT5G04900); *Oryza sativa Japonica* Group NOL (XP_015628274.1); *Oryza sativa Japonica* Group NYC1 (XP_015621887.1); *Klebsormidium nitens* NYC1 (GAQ77737.1); *Klebsormidium nitens* NOL (GAQ87774.1); *Coccomyxa subellipsoidea* C-169 NYC1 (XP_005652224.1); *Coccomyxa subellipsoidea* C-169 NOL (XP_005646276.1); *Chlamydomonas reinhardtii* NYC1 (XP_001697080.1); *Chlamydomonas 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); *Prochlorothrix hollandica* (WP_081599361.1); *Acaryochloris* RCC1774 (1) (WP_110987898.1); *Acaryochloris* RCC1774 (2) (WP_110986784.1); *Synechocystis* PCC6803 (WP_041428273.1).

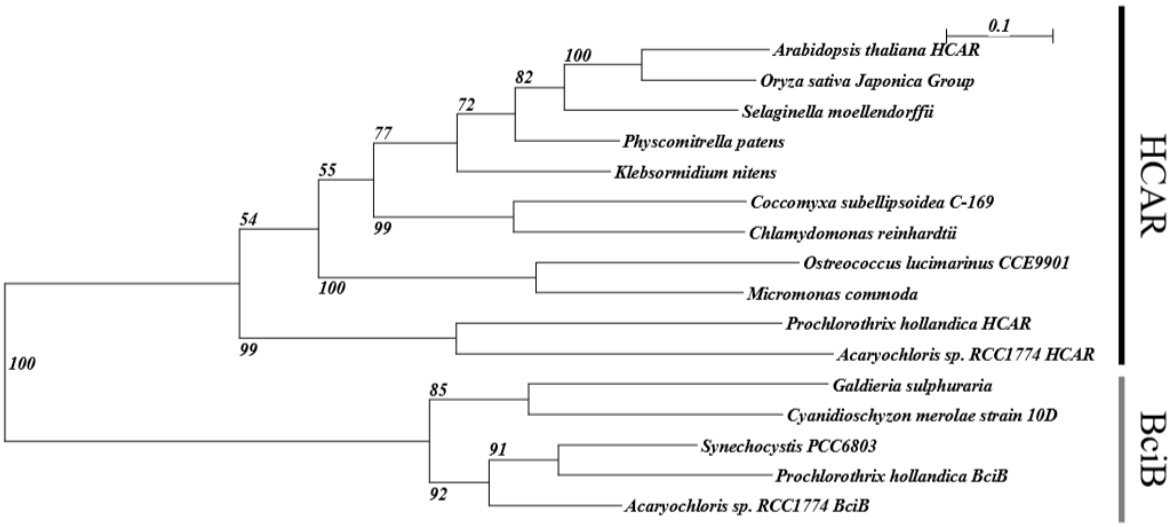

Figure 9. Phylogenetic tree of BciB and HCAR.

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. *Chlamydomonas reinhardtii* (PNW76723.1); *Micromonas commoda* (XP_002503439.1); *Ostreococcus lucimarinus* CCE9901 (XP_001416225.1); *Coccomyxa subellipsoidea* C-169 (XP_005648937.1); *Klebsormidium nitens* (GAQ88093.1); *Physcomitrella patens* (XP_024368500.1); *Selaginella moellendorffii* (XP_024538910.1); *Oryza sativa Japonica* Group (XP_015636785.1); *Arabidopsis* HCAR (AT1G04620.1); *Acaryochloris* RCC1774 HCAR (WP_110987361.1); *Prochlorothrix hollandica* HCAR (WP_017711629.1); *Acaryochloris* RCC1774 BciB (PZD72398.1); *Prochlorothrix hollandica* BciB (WP_044076442.1); *Synechocystis* pcc6803 (WP_010873198.1); *Cyanidioschyzon merolae* strain 10D (XP_005534820.1); *Galdieria sulphuraaria* (XP_005706147.1); *Chloroherpeton thalassium* (WP_012499756.1)

2.5 Enzymatic analysis of the candidate genes of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774

To clarify whether CBR homologues of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 have CBR activity or not, the recombinant proteins encoded by these genes were expressed in *E. coli*. Immunoblotting analysis showed that these proteins were successfully expressed in *E. coli* and found in a soluble fraction (Figure 10). *Acaryochloris* RCC1774 has two candidates, WP_110987898 and WP_110986784, for a potential CBR gene. When the recombinant protein of WP_110986784 was incubated with chlorophyll b, we detected two new peaks in the HPLC chromatograms for pigment analysis, neither of which were found in the negative control experiment (Figure 11). The retention times and absorption spectra of these pigments matched those of 7-hydroxymethyl chlorophyll a and its epimer form (Figure 12). Therefore, we concluded that *Acaryochloris* RCC1773 WP_110986784 encodes CBR. In contrast, we could not detect CBR activity with WP_110987898. In *Prochlorothrix hollandica*, we found one candidate gene, WP_081599361, for a potential CBR gene by phylogenetic analysis. We examined CBR activity of this recombinant protein and found that this gene has CBR activity. These results indicate that *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 have CBR genes. A large amount of the epimer type of 7-hydroxymethyl chlorophyll a was formed after incubation of chlorophyll a with *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 CBRs (Figure 11). In contrast, HPLC analysis showed that

chlorophyll a' was not accumulated in a large amount in cells (Figure 5), suggesting that production of this epimer form of 7-hydroxymethyl chlorophyll a by CBR is suppressed in cells.

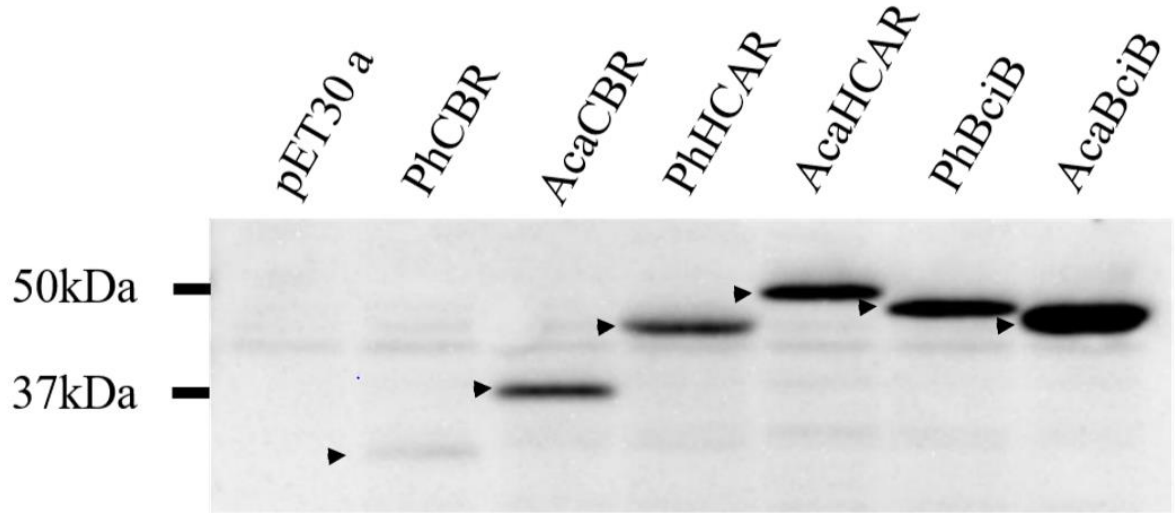

Figure 10. Immunoblotting analysis of recombinant proteins using a specific antibody against Histidine tag.

Candidate genes of CBR or HCAR were expressed in *E. coli* and soluble fraction of the cell lysate was analyzed by immunoblotting. The markers for molecular size are show in the left side. The cell lysate of *E. coli* having an empty vector (pET30a) was employed for the negative standard. Expressed proteins are indicated by the black arrowheads.

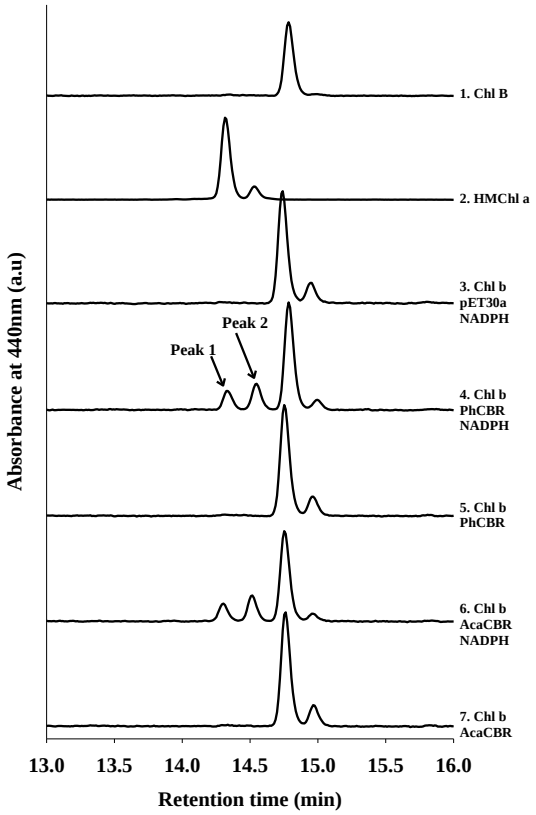

Figure 11. Enzymatic analysis of CBR of *Acaryochloris* RCC1774 and *Prochlorothrix hollandica*.

HPLC profiles of the pigments after incubation of chlorophyll with recombinant proteins. Chlorophyll b was incubated with the lysate of *E. coli* expressing AcaCBR and PhCBR with or without NADPH. 1, chlorophyll b; 2, 7-hydroxymethyl chlorophyll a; 3, lysate of *E. coli* having pET30a and chlorophyll b, incubated with NADPH; 4, lysate of *E. coli* expressing PhCBR and chlorophyll b, incubated with NADPH; 5, lysate of *E. coli* expressing PhCBR and 7-hydroxymethyl chlorophyll a, incubated without NADPH; 6, lysate of *E. coli* expressing AcaCBR and chlorophyll b, incubated with NADPH; 7, lysate of *E. coli* expressing AcaCBR and chlorophyll b, incubated without NADPH. Chl b, chlorophyll b; HMChl a, 7-hydroxymethyl chlorophyll a.

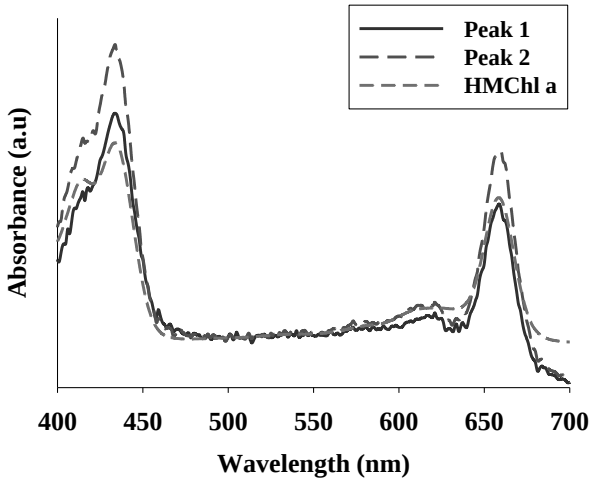

Figure 12. The comparing absorption spectrum

Comparing the 7-hydroxymethyl chlorophyll a, where peaks 1 and 2 were found in the reaction mixture of the lysate of *E. coli* expressing PhCBR and NADPH, as illustrated in Figure 11.

2.6 Catalytic specificity of HCAR

Phylogenetic analysis suggests that *Acaryochloris* RCC1774 WP_110987361 and *Prochlorothrix hollandica* WP_017711629 encode HCAR. Recombinant proteins of these genes were expressed in *E. coli*, and the cell extract was incubated with 7-hydroxymethyl chlorophyll a. HPLC analysis showed that 7-hydroxymethyl chlorophyll a was converted to chlorophyll a by these proteins (Figure 13 and 14), indicating that both *Acaryochloris* RCC1774 WP_110987361 and *Prochlorothrix hollandica* WP_017711629 encode HCAR. These results clearly show that *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 have the chlorophyll cycle.

Previous studies showed that HCAR and BciB show sequence similarity. A phylogenic analysis indicates that HCAR arose within the cluster of BciB during evolution. Interestingly, it was shown that cyanobacterial BciB has promiscuous HCAR activity (Ito and Tanaka 2014), which might have enabled the enzyme evolution from BciB to HCAR. In 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 (7-hydroxymethyl chlorophyll a) (Ito and Tanaka 2014). We hypothesized that promiscuous activity (such as HCAR activity in *Synechocystis*) 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 enzymes, *Acaryochloris* RCC1774 BciB (WP_110987361),

Prochlorothrix hollandica BciB (WP_044076442), *Acaryochloris* RCC1774 HCAR (WP_110987361), and *Prochlorothrix hollandica* HCAR (WP_017711629). BciB of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 could not convert 7-hydroxymethyl chlorophyll a to chlorophyll a (Figure 15 and 16), which is different from BciB of *Synechocystis* PCC6803. HCAR could not reduce divinyl chlorophyll a to monovinyl chlorophyll a (Figure 13). HCARs of *Acaryochloris* RCC1774 and *Prochlorothrix hollandica* have no 8-vinyl reductase activity (Figure 13). These results support our hypothesis indicating that these enzymes have evolved to acquire strict substrate specificity.

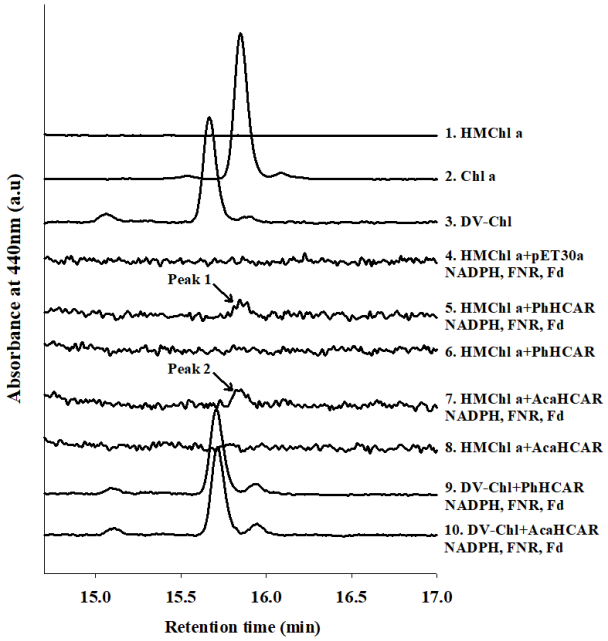

Figure 13. Enzymatic analysis of HCAR of *Acaryochloris* RCC1774 and *Prochlorothrix hollandica*.

Various activities and substrate specificity of two different HCAR using *E. coli* lysate. After terminating the incubation, the pigment changes were inspected by HPLC. The spectrum chl a and peak 1 were compared in B. 1, HMchl a; 2, Chl a; 3, DV; 4, pET30a and HMChl a with NADPH, FNR and Fd; 5, PhHCAR and HMChl a with NADPH, FNR and Fd; 6, PhHCAR and HMChl a without reductases; 7. AcaHCAR and HMChl a with NADPH, FNR and Fd; 8, AcaHCAR and HMChl a without reductases; 9, PhHCAR and DV with NADPH, FNR and Fd; 10, AcaHCAR and DV with NADPH, FNR and Fd.

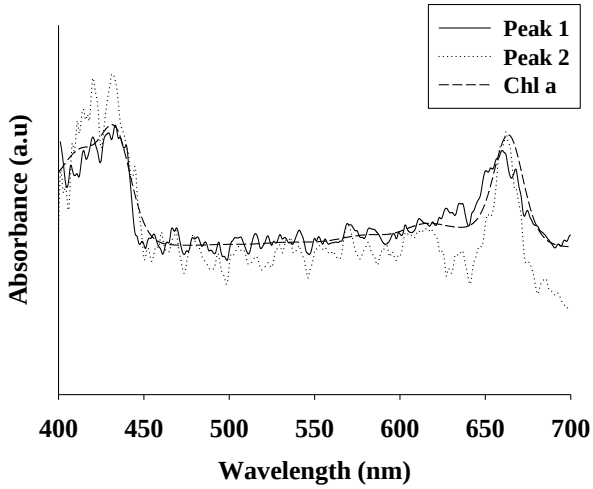

Figure 14. The comparing the absorption spectrum

Comparing chlorophyll a, where peaks 1 and 2 were found in the reaction mixture of the lysate of *E. coli* expressing PhHCAR and AcaHCAR with reductant, as illustrated Figure 13

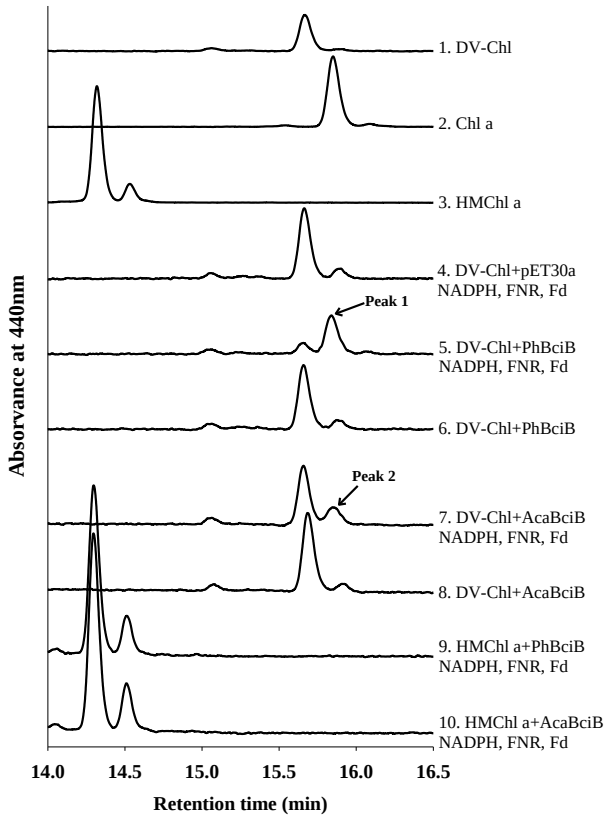

Figure 15. Enzymatic analysis of BciB of *Acaryochloris* RCC1774 and *Prochlorothrix hollandica*.

HPLC profiles of the pigments after incubation of chlorophyll with recombinant proteins. divinyl chlorophyll a and d 7-hydroxymethyl chlorophyll a were incubated with the lysate of *E. coli* expressing AcaBciB and PhBciB with or without NADPH, FNR and Fd. 1, divinyl chlorophyll a; 2, chlorophyll a; 3, 7-hydroxymethyl chlorophyll a; 4, lysate of *E. coli* having pET30a and divinyl chlorophyll a, incubated with NADPH, FNR, and Fd; 5, lysate of *E. coli* expressing PhBciB and divinyl chlorophyll a, incubated with NADPH, FNR, and Fd; 6, lysate of *E. coli* expressing PhBciB and divinyl chlorophyll a, incubated without reductant; 7. lysate of *E. coli* expressing AcaBciB and divinyl chlorophyll a, incubated with NADPH, FNR, and Fd; 8, lysate of *E. coli* expressing AcaBciB and divinyl chlorophyll a, incubated without reductant; 9, lysate of *E. coli* expressing PhBciB and 7-hydroxymethyl chlorophyll a, incubated with NA DPH, FNR, and Fd; 10, lysate of *E. coli* expressing AcaBciB and 7-hydroxymethyl chlorophyll a, incubated with NA DPH, FNR, and Fd. DV-Chl, divinyl chlorophyll a; Chl a, chlorophyll a ; HMChl a, 7-hydroxymethyl chlorophyll a.

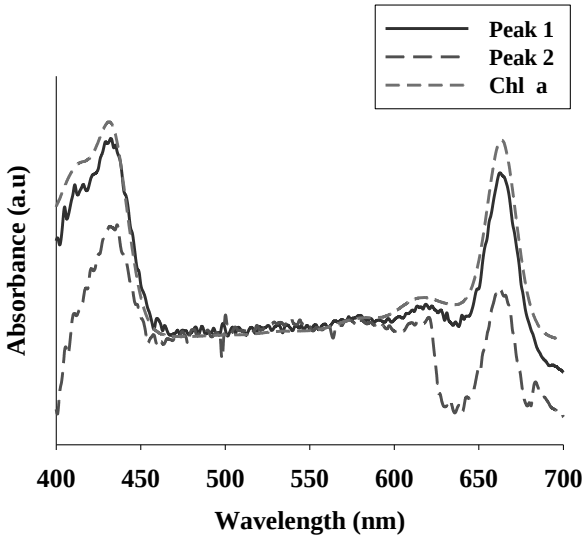

Figure 16. The comparing absorption spectrum

Comparing chlorophyll a, where peaks 1 and 2 were found in the reaction mixture of the lysate of *E. coli* expressing PhBciB and AcaBciB with reductant, as illustrated in Figure 15

3 Discussion

3.1 Chlorophyll cycle in cyanobacteria containing chlorophyll b

We showed in this report that cyanobacteria containing chlorophyll b, *Prochlorothrix hollandica* and *Acaryochloris* RCC1774, have the chlorophyll cycle consisting of three enzymes, CAO, CBR, and HCAR as in green plants. *Acaryochloris* RCC1774 and *Prochlorothrix hollandica* grow in shallow seas and fresh water, respectively, where light conditions change dynamically, suggesting that these two organisms need to control the antenna size in response to light conditions, as in green algae. In *Prochlorothrix hollandica*, chlorophyll b is localized in chlorophyll a/b-binding proteins (encoded by PCB genes) which are not related to LHC (Bumba, Prasil, and Vacha 2005; Herbstová et al. 2010). The antenna size and chlorophyll a/b ratio are dynamically changed in response to light intensity in *Prochlorothrix hollandica* (Burger-Wiersma and Post 2008). However, the mechanism of antenna size regulation is different between *Prochlorothrix hollandica* and green plants, because the chlorophyll a/b ratio becomes low under high light conditions in *Prochlorothrix hollandica* (Burger-Wiersma and Post 2008) but high in green plants. Although the mechanism is different, the chlorophyll a/b ratio must be regulated in *Prochlorothrix hollandica*. The chlorophyll cycle would contribute to this regulation. There are no reports concerning the changes in chlorophyll a/b ratio in *Acaryochloris* RCC1774. It would be reasonable to hypothesize that the chlorophyll a/b ratio changes in response to light conditions, because some *Acaryochloris* species contain another antenna

pigment, chlorophyll d, and they change chlorophyll d/a ratios in response to light conditions (Duxbury et al. 2009). Chlorophyll b to a conversion is an important step during leaf senescence in plants (Kusaba et al. 2007), because the chlorophyll-degrading enzymes of plants cannot catabolize chlorophyll b-type pigments that contain a formyl group at the C7 position. It is possible that the chlorophyll cycle could also play an important role in chlorophyll b degradation in cyanobacteria containing chlorophyll b. The physiological functions of the chlorophyll cycle in cyanobacteria with chlorophyll b remains to be studied.

We could not find any genes that show sequence similarity to either HCAR or BciB in Prochlorococcus. As mentioned above, BciB catalyzes the conversion of divinyl chlorophyllide to monovinyl chlorophyllide. Prochlorococcus uses divinyl chlorophyll instead of monovinyl chlorophyll (Barrera-Rojas et al. 2018), therefore, it is reasonable that Prochlorococcus lacks BciB. Our analysis presented in this study indicates that Prochlorococcus does not have the chlorophyll cycle either. It is reported that Prochlorococcus has evolved into high-light and low-light-adapted ecotypes (Rocap et al. 2003), which have genetically adapted to different light niches. Low-light-adapted Prochlorococcus ecotype is found in deep seas. It has a large number of PCB genes and a low chlorophyll a/b ratio. In contrast, high-light-adapted Prochlorococcus has a high chlorophyll 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 (Garcia-Fernandez, de Marsac, and Diez 2004),

which might also account for the absence of the chlorophyll cycle in Prochlorococcus.

One potential drawback to losing the chlorophyll cycle might be the loss of the ability to degrade chlorophyll b. This is because one of the enzymes of chlorophyll degradation pathway (pheophorbide a oxygenase) does not catalyze the substrate if the pheophorbide has a formyl group on C7 position (Hörtensteiner, S., Vicentini, F., & Matile 1995). At present, it is not clear whether Prochlorococcus has an alternative chlorophyll b degradation pathway or if it just excretes chlorophyll b outside the cell after conversion to chlorophyllide b. Further studies are needed to elucidate chlorophyll degradation in Prochlorococcus and other cyanobacteria.

Acaryochloris containing chlorophyll b has the chlorophyll cycle by which chlorophyll a and chlorophyll b are interconverted. Other *Acaryochloris* have chlorophyll d instead of chlorophyll b. These *Acaryochloris* are expected to have the interconversion pathway of chlorophyll a and chlorophyll d, which would be beneficial for altering the chlorophyll 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 chlorophyll biosynthesis (G. E. Chen et al. 2016). Identification of the enzymes responsible for the chlorophyll a and chlorophyll d interconversion is indispensable for understanding the acclimation of *Acaryochloris* containing chlorophyll d.

3.2 Evolution of the enzymes of the chlorophyll 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* PCC6803 has 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 (7-hydroxymethyl chlorophyll a) for this promiscuous activity in the cell. This broad substrate specificity and catalytic activity would contribute for the enzyme to have high catalytic activity of the primary reaction because high specificity accompanies the low catalytic activity (Khersonsky and Tawfik 2010). In contrast with BciB of *Synechocystis* PCC6803, BciB of *Prochlorothrix hollandica* and *Acaryochloris* RCC1774 have no HCAR activity. It should be noted that chlorophyll synthesis and chlorophyll b to chlorophyll a conversion are regulated differently because the former is responsible for the synthesis of chlorophyll a and chlorophyll b, but the latter is involved in the degradation of chlorophyll b. If BciB were to have HCAR activity, the regulation of chlorophyll metabolism would be disturbed. This might be the reason why BciB have evolved to have high substrate specificity in *Prochlorothrix hollandica* and *Acaryochloris* RCC1774. The same phenomenon is observed with HCAR of the green plants and cyanobacteria containing chlorophyll b. These HCARs have no BciB activity because these organisms have another 8-vinyl reductase. These results are consistent with the hypothesis of Khersonsky and Tawfik (2010) mentioned above, that is, the assumption that if a promiscuous activity is harmful, the enzyme evolves

to lose this activity. In contrast, if the promiscuous activity is not harmful, the enzyme retains its promiscuous activity to increase the primary activity.

Our phylogenetic analysis indicates that BciB was duplicated or horizontally transferred from other organisms and evolved to acquire chlorophyll b reductase activity in *Prochlorothrix hollandica* and/or *Acaryochloris* RCC1774. It is not evident whether a common ancestor of cyanobacteria containing chlorophyll b had HCAR. In this case, HCAR would have been vertically inherited by the contemporary cyanobacteria containing chlorophyll b. Otherwise, it is also possible to hypothesize that one cyanobacterium acquired HCAR and laterally transferred to the other bacteria containing chlorophyll b. HCARs of cyanobacteria and green plants form a single cluster, suggesting that green plant HCAR evolved from cyanobacterial HCAR. Considering that the tree topologies are similar between CAO and HCAR, 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 (Figure 8). 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 a gene homologous to green plant CBR. If the phylogenetic tree of CBR is correctly reconstructed, the tree indicates that CBR homologues of red and brown algae are evolved from CBR. This also suggests that the common ancestor of eukaryotic photosynthesis organisms had chlorophyll b. We also found homologues in green filamentous photosynthetic bacteria. Elucidation of

the function of these CBR homologues would be helpful for understanding enzyme evolution.

4 Materials and Methods

4.1 Phylogenetic tree analysis

The protein sequences were obtained from the data bases 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 (Table 1 and Table 2). 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.

Table 1. The trimmed sequence list of CBR

*Arabidopsis*_NYC1	AGPRNVVITGSTRGLGKALAREFLLSGDRVIVTSRSSESVDMTVKELE---- QKVVGIACDVCKPEDVEKLSNFAVKELGSINIWINNAGTNKGFRPLLEFTEEDI TQIVSTNLIGSILCTRGAMDVMSRQHGHIFNMDGAGSGGSSTPLTAVYGSTKC GLRQFHGSIVKESNVGLHTASPGMVLTELLLSGIICELPETVARTLVPRMR
WP_110987898.1 [*Acaryochloris* RCC1774 (1)]	LQGRTAVVTGSTRGIGLIMAQALIAAGANVVVSSRSETGA--VCEQLER- NALQIMGTTCDVTSLGQVEQLAAKTIERFGQIDIWINNAGIASPYAQTLDIPIER WKRVVQTNLYGTYHGTTVALQHMLERNGKIVNVFGAGDRDSVYGYMSAYAT SKSAVRRFTLVMAEEYPISILGLRPGLVATDLMTKILFTTPAEQIQTTIVKMVS
WP_081599361.1 [*Prochlorothrix hollandica*]	LQHRTVVITGSTRGIGLIMAQAMAEAGSNVVISSRSQGAIAAVLPQL----- QQVLGVPCDITDFDQVQQLGQRTLDRFGSVDVWFNNAATTCPFGPVLDIPMA QWRQVIETNVIGTYHGTTVALERMLPQGGTIINLLGAGTNDTANGYLSAYTAS KAAVQRFTQVAADDYGIKVCGFNPGLVPTDLTLKIWLSTDPTAIASRSVSLAI
WP_110986784.1 [*Acaryochloris* RCC1774 (2)]	LQDRVAVVTGSTQGIGLIIAQALAAAGAKIVICSRSESAVEAACSQL----- EQVFGLPCDVADPEQVENLAQQTLERFGQIDVWFNNASVNRYFGPALDLPIDH WHEVINTNLNGAYYGTMTALRHMLPRDGKIINMLGAGAQDSGDSYLSAYATS KAAARRLTLVVAEDYGISVLGMNPGLFSTQLTTKIWLATSPETIAQTAVRVAS
WP_041428273.1 [*Synechocystis* PCC6803]	LDGKVAVVTGASKGIGAEIAKHLAGEGAAVVVNYSSKEGADRVVDEI------ KAIAVQANVAKKAEIQQLFAETKQAFGKLDILVNNAGI-- YFSPLEGITEEHFYKQFDLNVLGLLLTSQQAVKSFGEE- GSIINISSIVSTLTPANSLV- YNATKAAVDAITKSLAKELNIRVNSINPGMVETEGRTAG-------RQVEAITPLGR
OLS31532.1 [*Heimdallarchaeota archaeon* AB_125]	---MKVVITGSSKGIGYALAKEFAKYGDQIVISSRNQDSVDKAVEEIK---- NKVHGTTCNVSKPEEIKNLISFSDENLSGIDIWINNAGINGSYGNLTTWENETL DSVIQTNVLGTLYGCKEAISYMTNQGGKIFNLAGMGSNGMASPKMVVYGAS KASIPQLTKSLSKELNILINYLSPGIVITDFIIN-ILGEKPDKVAKFLVRKIY
*Arabidopsis*_NOL	TPPYNILITGSTKGIGYALAREFLKAGDNVVICSRSAERVETAVQSLK---- EHVWGTKCDVTEGKDVRELVAYSQKNLKYIDIWINNAGSNAYFKPLAEASDE DLIEVVKTNTLGLMLCCREAMNMMLTQSGHIFNIDGAGSDGRPTPRFAAYGAT KRSVVHLTKSLQAELNVVVHNLSPGMVTTDLLMSGVLAEPAEVVAEYLVPNI R
XP_015628274.1 [*Oryza sativa Japonica* Group NOL]	VPPYNVLITGSTKGIGYALAKEFLKAGDNVVICSRSAERVESAVTDLK---- KHVWGIVCDVREGKDVKALVDFARDKMKYIDIWINNAGSNAYYKPLVETSDE ALMEVITTNTLGLMICCREAINMMRNQPGHIFNIDGAGSDGRPTPRFAAYGAT KRSVVHLTKSLQAELNVMVHNLSPGMVTTDLLMSGILAEPANVVADYLVPNI R
GAQ87774.1 [*Klebsormidium nitens* NYC1]	SPPYNVVITGSSKGIGLALAKQFLAAGDRVCLCARDTSQLEAQCKEFE---- KQTLAWATDVTKAAEVADLANFAKQAMGHVDVWINNAGTNAYYKPLVENS DEDIEQIVATNTLGVMLCCRQAIKLMQKQGGHIFNMDGAGADGNPTPRFAAY GATKRGLAQFTKSLQAELNVTVHNLSPGMVTTDLLMAGALAEPPDVVADFLV PRIR

XP_005646276.1 [*Coccomyxa subellipsoidea* C-169 NOL]	QPPYNVVITGGTKGVGRALAKEFLRAGDSVVICSRDSDRVNGTVRELDGFSK ARIKGKVCNMAKPGDVASFANYARDTLGTVDLWINNAGSNGYYKTLAESSD ADLINIVETNVLGTMLGCKEAIRVMRDQRGHIFNMDGAGADGGATPRFAAYG ATKRGLMQLSKSLQAELNVGIHNLSPGMVTTDLLMAGCLADPPEEVAAYLVP RIR
XP_001701347.1 [*Chlamydomonas reinhardtii* NOL]	QAPYNVVITGSTKGIGRALAEDFLRAGDRVVVCSRTGDRVSETVAELA---- ARVKGLAVDVSAPGQARQLADFAAQELGRVDIWINNAGTNAYYGPMAESTD EELSQIVGTNVLGVMLCCKEAIRVMRSQSGPL-----T--- GALQPVYIRFLTQGKALQRVAA------ NVAVHNLSPGMVTTELLMAGCLVQACGGAGGQDRPGAG
CEM24690.1 [*Vitrella brassicaformis* CCMP3155]	TTSKNVVITGGTKGVGYALAKRFLREGDRVIVCGRDADRLAMAVEALR---- RSVGGILCDVGSPQDVERLGDFCVEQLGHIDVWVNNAGTVAGRRRLSDLTPD DLKQVLDTNLLGTLLCCKKAIEIMSRQEGHIFNMDGAGVEGGATKGYAAYGA SKRAMPQLSASIREELKIGVHNLSPGMVLTDLLLADTLCEEPDTVADYLVPRIR
GAX23003.1 [*Fistulifera solaris*]	MPDGGVVITGAAGGVGFAYAGEFMDRGYDVVIC--DVRDCKMAADALA---- RKVFHTKCDVSDSKEVEKLGEFAKSKLGTIGYWINNAGVNGGRRELRDVPVS QVELVVKVNLLGILLCTKVAMSIMEQQAGHIFNTVGSGVKGGGTPGYACYGA TKRGLPQLTASLVKELKIMVHNLSPGMVFTKLLLDDVLAAQPEEVAADLVPKI L
XP_005716045.1 [*Chondrus crispus*]	SSSLGVVISGSTKGVGRALAEEFVKQNDGVVISSRTPDSVDSTVASLR---- RRVFGCVADVSKHLDVARLADFASENLGTINTFICNAGTTGPRGPIRDAEANDL ANVVSTNLLGPMLCAKEAWRVAKNQSLHVFIMDGSGSRGNTTPNYAAYGATK RSIPQLVASLAIEGPVRFHTLSPGMVLTDLLLAEFLAEEPETVAENLVPRIR
WP_010932815.1 [*Chlorobaculum tepidum*]	RKSLGVVITGGSAGLGLAMAREFLRAGDRVVICSRRESNLKSALQMLG---- SNVYGMVCDVSLPAQAADFAAFAAAKLGIIDRWINNAGTAGRRRPLWELDLS DIDETCRTNLSGSMMLCAEALRVMLRQPYHLFNMGFSSAGLRSSPTSVPHRAS KRAVAIMSKLLRQELSVGIHELSPGLVLTDLLLRDAMAETSETVAATLVPAIR
WP_069809375.1 [*Chlorobaculum limnaeum*]	SASLGVVITGGTAGLGLAMAREFLRAGDRVVVCSRRDSNLALALQTLE---- REAHGMACDVSDPRQAAEFAAFAAGKLCVIDRWINNAGTAGRRRPLWELDLT DIDETCRTNLSGSMMLCSEALRVMLRQPYHIFNMGFTAAGLHSSPTSVPHRAS KRAVAIMSELFRQELSVGVHELSPGLALTDLLLRDAMAETPETVAAKLVPMIR
XP_005652224.1 [*Coccomyxa subellipsoidea* C-169 NYC1]	HAPLNVVVTGGTRGIGKAIAREFLRSGDRVMVSSRSVQAVRRAMSELR---- EWIGGIDCDVSSPASVQRLVDGAASQMGSIDVWINNAGYSGTFQSFIEARPEQI QEVVQTNLLGCLLCTRAAMRLMAAQPGHIFNMDGAGADGLPTPQYAAYGAT KAGIAHLKGSLGAEAPVGVHCLSPGMVLTNLLLEGILCEQPETVAAFLVPRAR
XP_001697080.1 [*Chlamydomonas reinhardtii* NYC1]	RQPLTVVVTGGSRGLGKALAREFLAAGDRVLLTSRTQAAADAAVRELR---- EQVVGVAADVSDAVGVAAVEAAALSSFGRVDAWVNNAGYSGSFQPLVEQTD AQIEQVVRTNLLGTLLCTRQAVSLMQHQPGHIFNMDGAGADGFATPNYAAYG ATKAGITQLTGTLQRELPIKLHTVSPGMILTDLLLEGILCEHPETVAAFLVPRIK

GAQ77737.1 [*Klebsormidium nitens* NYC1]	STQRNVVVTGSTRGLGKALAREFLRFGDNVVVASRSEEAVRRTVAELR---- KRVVGRACDVSDSADVRRLAEFAQSNFGHVDLWINNAGQNPGAKSLMEFED EEISSVVATNLVGSLVCTKEAIRFMRSQPGHVFNMDGNGSGGNATPQYAVYGA TKCALRQLQQTLLRETKVGVHTASPGMVLTELLLAGFICEQPETVAKALVPRL R
XP_015621887.1 [*Oryza sativa Japonica* Group NYC1]	AGPRNVVITGSTRGLGKALAREFLLSGDRVVIASRSPESVLQTINELE---- EKVVGTSCDVCKPEDVKKLVNFAKDELGSIDIWINNAGTNKGFRPLVNFSDED ISQIVSTNLVGSLLCTREAMNVMQHQQGHVFNMDGAGSGGSSTPLTAVYGST KCGLRQFQASLLKESKVGVHTASPGMVLTDLLLSGLICELPETVARTLVPRMR

Table 2. The trimmed sequence list of HCAR

PNW76723.1 hypothetical protein CHLRE_11g468700v5 [*Chlamydomonas reinhardtii*]	AKPIQPGSSYPAKEHCSNCGLCDTYYVAHVKDACAFLGPGMSRIDELEERVHG RRRDVNSDDELHFGVTAGGMAYAANVPGVPGAQWTGIVTQIAIEMLQSGKV DAVVCVQSDENDRFTPKPVVARTVEDIIKARGVKPTLSPNLNVLATVEALQVK KLLFIGVGCQVQALRSIEPHLGLEKLYVLGTNCVDNGPRKGLDKFLKAASTRP DQALHYEFMQDYRVHVKHTD---GS-- FEYVPYFCLPANELNDVIAPSCYSCFDYPNALADMVVGYMGVPLNKDMTSHP QYVVVRNDRGNELLDSVRH- RLQITPTVSTGDRRGIVMQTVASDDEAMGQLRDPAPRWLGNMLAWLLNLIGP KGLEFGKYSIDYHYIRNYLYVNRKWGAKRAEQHIPSFAKKIVQQYDKDGAVS KRISL
XP_002503439.1 predicted protein [*Micromonas commoda*]	SKPIAPGGNYPAKEHCSQCGLCDTYYIAHVKDACAFLGDGMSRIETLEPTVHG RGRDLG-NDEMRLGVVD- EVFYAKRNRPVEGAQWTGIVTSIAIEMLKSGKVEGVVCVASDPDNAMHPRPIL ATTVEEILSSKGVKPALSPNLSVLAEVEARGLKRVLFIGVGCAVQALRSVEKYL GLEKLYVMGTNCTDNGRKETLSKFLENASEDPATVVHYEFMQDYQVHLKHT D---GS-- FEKVPYFCLPANKLKDVIAPSCYSCFDYVNGLADIVVGYMGVPYHTDMTRHP QYVTVRNERGKEMFDMIRG- DCDVTPSVSSGERKPFVMQTVISDDEALGRPEEPAPLPVGKAIAWLLEKIGPKG KEFGMYSLDYHTIRNYLYVKRTFGEERATRHVPDYARLVVDEYNVYGAVDER LKL
XP_001416225.1 predicted protein [*Ostreococcus lucimarinus* CCE9901]	ARPIAPGSAYPAKEHCSECGLCDTAHVARVKEACAFLGPGQSRIETLEPVVHGR ARSAAPSDESRLGVAL- ETFYGAMRTPVDGAQWTGIVTSVALAALRSGAVEGVVCVASREDDSRAPRPIL ATTEEEILSARGVKPSLSPNLSVLAEVEARGLKRVLFIGVGCAVSALRAVEPHL GLDALYVVGTNCTDNGRWEGFNKFIDAASDDPDTVMHYEFMQDYQV---------- ------- PYFCLPAKDLTDVIAPSCYSCFDYVNGLADVVVGYMGVPMDKPMDRHPQYV TVRNERGREMIDLIRN- DMEITPSTSSGDRRPFVMQTVVADDEALGRPDKPAPRVVGKLLAWLLTKIGPK GKEFGMYSLDYHTIRNYMYVNRAWGAKRAEEHVPEYAKRVVREYDVDGAIS ARLRL
XP_005648937.1 hypothetical protein COCSUDRAFT_14021 [*Coccomyxa subellipsoidea* C-169]	AKPIKEGSTYPAKQFCSHCGLCDTYYVAHVKDACAFLGDGMSKIESLEEQVH GRRRDLDSLDDLHFGVHE-- MMYALNTPPVPGAQWTGIVTQIAIEMLESGQVEAVVCVQNDEADRFSPKPFV ARCKEDILKAKGVKPTLSPNLNTLATVEALDVKRLLFIGV---- QALRSVEKYLGLEALYVLGTNCVDNGPREGLEKFLNAASSDPDTVLHYEFMQ DYRVHIKHLD---GS-- FEYVPYFCLPANDLTDVIAPSCYSCFDYPNALADLVVGYMGVPYGTDMTSHP QYITVRNARGRAMMDAVKP- RLKILPTMAAGDRTPFVMQTVLSDDAGLGTAPNPAPRFVGNAIAKVLTWLGP KGLEFGRYSIDYHYIRNWIYVNRHMGPARAQRHTPEFAKRLVAMYNEKGEID AR---

GAQ88093.1 7-hydroxymethyl chlorophyll a reductase [*Klebsormidium nitens*]	ARPIQPGSAYPAKEHCSNCGLCDTYYVAHVKDACAFLGDGMSRVEDLEPRVH GRGRDPDSLDDAHFGVHK- EMLYAKKTNPIEGAQWTGIVTGIAMEMLRSGKVDAVVCVQSDPEDRFKPKPV LARTPEEVLAARGVKPTLSPNLNVLALVEAAGVKRLLFCGVGCQVQALRSVE QHLGLEKLYVLGTNCVDNGPRAGLEKFLNAASDEPETVLHYEFMQDYKVHL KHLD---GR-- NEEVPYFCLPADDLKDVIAPSCYTCFDYTNGLADLVVGYMGVPEGVPMTRHP QYVTVRNDRGAEMLDLVRN- QLEITPTVSSGDRKSFVLETVKADDKAIGLTTVPAPLFIGNAIAWVLDKIGPKGL EFGRYSIDYHFIRNYLYVVRTWGPKRAAQHIPAYAQRLVNMYNKKGEIDRILD E
XP_024368500.1 7-hydroxymethyl chlorophyll a reductase, chloroplastic-like [*Physcomitrella patens*]	AKPIKPGSTYPAKDHCSQCGLCDTYYIAHVKDACAFLGDGMSRIEVLEPKVHG RGRNPESMEDLFFGVHD- EMLYARKTEPVEGAQWTGIVTTIAMEMLRKDMVDAVICVQSDPEDRFKPNPV LARTPEEVLAARGVKPTLSPNLSTLAFVEAAGVKRLLFCGVGCQVQALRSVE KHLGLEKLYVLGTNCVDNGPRQGLDKFLKAASDDPDTVLHYEFMQDYKVHL KHLD---GR-- NEEVPYFCLPADDLTDVIAPSCYSCFDYTNGLADLVVGYMGVPPGVPMTRHP QYITVRNGRGKEMLDLVRP- LLDVTPTISSGNRGPFVMETVKADDKALGKKTQPAPRFVGNIIAWLLNLVGPK GLEFGRYSLDYHNIRNYLHVHRAWGQKRADQHIPSYAKKLVSLYNKNGEIDKI LED
XP_024538910.1 7-hydroxymethyl chlorophyll a reductase, chloroplastic-like [*Selaginella moellendorffii*]	SKPIKPGSTYPAKEFCSHCGLCDSYYIAHVKKACAFLGNGMTKVEAMEPEVH GRARDVKSLDELYFGVHE- ELLYARKIEPVKGAQWTGIVTAIAIEMLKTKRVEAVICVQSDPEDRFTPRPVLA RTPEEILAARGVKPTLSPNLNTLALVEAAGVKKLLFCGVGCQVQALRAVEKYL GLEKLYVLGTNCVDNGPREGLDKFLRAASDSPQTVLHYEFMQDYKVHLKHL D---GR-- MEEVPYFCLPASDLTDVIAPSCYSCFDYTNGLADLVVGYMGVPPGVSMVQHP QYVTVRNERGREMLDLVKH- LLEVTPTVSTGDRRPFVMETVKADDNALGLKSAPAPRFVGNIIAFLLNLIGPKG LEFGRYSLDYHTIRNYLYVQRAMGKSRAEAHIPSYSKELVEKYNEGGAIDKLL KR
XP_015636785.1 PREDICTED: 7-hydroxymethyl chlorophyll a reductase, chloroplastic isoform X3 [*Oryza sativa Japonica* Group]	SKAIPPGGVYPAKDHCSQCGLCDTYYIAHVKNACAFLGDGMSRVEDLEPLVH GRGRKQD-MDEMYFGVYE- QLLYARKMKPVEGAQWTGIVTTIAVEMLKANMVDAVVCVQSDPDDRLAPMP VLARTPDEVIAAKGVKPTLSPNLNTLALVEAAGVKRLLFCGVGCQVQALRSV EKYLGLEKLYVLGTNCVDNGTREGLDKFLKAASSEPETVLHYEFMQDYKVHL KHLD---GH-- IEEVPYFCLPAKDLVDVIAPSCYSCFDYTNGLADLVVGYMGVPPGVSMTQHPQ YITVRNDRGREMLSLVEG- LLESTPTVSSGVRQPFVIETVKADDEAQGRPSQPAPTFVGNVIAFLLNLIGPKGL EFARYSLDYHTIRNYLHVNRAWGKQRAEQHIPSYAKKIVEAYDKDGRIESMLQ -

HCAR *Arabidopsis* AT1G04620.1	SRPIPPGGTYPAKDHCSQCGLCDTYYIAHVKEACAFLGDGMSRIESLEPVVHG RGRKADSLQDTYFGVHQ- EQLYARKLKPVEGAQWTGIVTTIAIEMLKSNMVEAVVCVQSDPEDRLSPRPVL ARTPEEVLAARGVKPTLSPNLNTLELIEASGVKRLLFCGVGCQVQALRSVEQH LNLEKLYVLGTNCVDNGTRDGLDKFLKAASKEPETVLHYEFMQDYKVQLKH LD---GH-- IEEVPYFSLPANDLVDVIAPSCYSCFDYTNALADLVIGYMGVPSGLNMTDHPQ YITVRNERGKEMLSLVEN- LLEITPTISSGDRRPFVTETVKADDAAFGQPAQPAPLFVGNIIAFILNLVGPKGLE FARYSLDYHTIRNYLYVNRKWGKQRANTHMPSYAKKIVEMYNKNGQIDKML SK
WP_110987361.1 hypothetical protein [*Acaryochloris sp.* RCC1774]	SQPIPPGGRYPAGDYCSHCGLCDTYYVAHVKDACAFLGDGMQKVETLEPQVH GRSRKDN--LERRFGVCT- AMHTVKMDPPVAGAQWTGVVTSLAIALLENDWVDGVICVQSDPDDRFKPKP VIATTVEEIMAARGVKPTLSPNLNILALLEESNLKRILFCGVGCQVQALRSVEH HLNLEQLYVIGTHCVDNGKREGLDKFLETTSDSPETVKHYEFMQDYQVHLKH TD---GH-- TEKVPYFCLPTKELNNVIAPSCYSCFDYMNGLADLVVGYMGVPQNVPMTEHY QQVTIRNKKGVQMFELIKP-KASIESVEMEGDCRNFVLQTVFQEE---- RSNSRLPKFIGKWLAAALTKFGPQGLEFAKYSIDYHTIRNYLFVKRHWGTK- ADQHIPGYSKAIVKEYDQNDKISRLLR-
WP_017711629.1 hypothetical protein [*Prochlorothrix hollandica*]	SRPIAPGSAYPAGDHCSHCGLCDTYYVAQVKTACAFLGEGMAKVERLESVVH GRDRHP---EERHFGVTH- QLTYGQVRQPVAGAQWTGLVTTIAVEMLRRGLVEGVVCVQSAAADARAPQP VLATTIEDIMAARGVKPTLSPNLNILETLESSGLKRILFCGVGCQVQALRSIEPQ LNLEKLYVLGTHCVDNGPRSGLEKFLRVASEHPETVQHYEFMQDYRVHFKHQ D---GS-- YERVPYFCLPAGELGDVIAPSCYSCFDYMNGLADLVVGYMGVPQHKPMTQH CQQVLVRNDRGQEMLDLVQP-LLALQAPESGGDRRNFVMQTVLQEE---- RSSQTLPKPLGHLLAWLLTKLGPQGLEFARYSIDYHTLRNYLYVARHRGSE- GLAQIPEYARAIVADYDQQGQIRQLLG-
WP_110987115.1 hypothetical protein [*Acaryochloris sp. RCC1774*]	--GLKRSSPRPAKALCSDCGLCDTYYIHYVKEACAFLN--- QQVAELEQQAHGQSRDLDSEDDWYFGVHQ- QMLTARKKDPIPGAQWTGIVSTIGIEMLNRGLVEGVVCVQNTPEDRFQPMPVI ARTPEDILAARVNKPTLSPNLSILEQIEQSGLKRLLVIGVGCQIQALRSVQDKLG LEKLYVLGTPCVDNVPRDGLQKFLETTSKSPETVVSYEFMQDFRVHFKHED---- ----EQVPFFGLKTDKLKNIF
WP_044076442.1 hypothetical protein [*Prochlorothrix hollandica*]	--GLKPGSRRPAKDLCSECGLCDTHYIHYVKDACAFLN--- QQFDQLEEKSHGRQRNLEDPRELYFGVHQ- TMVAARKKEPIPGAQWTGIVSTIACEMLTQGLVEGVVCVQNREDDRFGPQPV LARTPAEVLAAKVNKPTLSPNLSVLEQIEQSGMKRVLAIGVGCQIQALRAVQD QLGLEKLYVLGTPCVDNVSRAGLQKFLETTSFSPETVVHYEFMQDFNIHFKHE D--------EKVPFFGLKTNVLKDIF

WP_010873198.1 MULTISPECIES: hypothetical protein [*Synechocystis*]	--GLKPGSPRPAKELCSDCGLCDTYYIHYVKEACAFIN--- QQFDHLEEQTHGRSRELGKEDEVYFGVHQ- KMLTAQKKEPIPGAQWTGIVSTIGCEMLNKGLVEGVVCVQNTPEDRFQPQVVI ARTPAEVLAAKVNKPTLSPNLSVLEEVEKSGLKRLLVIGVGCQIQALRAVEKQ LGLEKLYVLGTPCVDNVSRAGLQKFLETTSRSPETVVYYEFMQDFRVHFKHE D--------ELVPFFGLKTNQLKEVF
XP_005534820.1 similar to coenzyme F420-reducing hydrogenase, beta subunit [*Cyanidioschyzon merolae* strain 10D]	LGGAYDTKVYPAKSLCSHCGLCDTRYITYVRDSCAFLN--- QHIAELEQKVHGRSRALDSEDELYFGVFQ- SMLVARKKKPIAGAQWTGIVSSLAIAMLESGLVEGVVCVQSSKDDRFKPVPVI ARNRAEILAARVNKPTLSPNLSVLDAVERSGIKRLLFIGVGCQVEALRSVQDRI GLEKLYVLGTPCVDNVTRAGLQKFLDTTSSSPETVVYYEFMQDFRVHFKHDD GGPGRKWDEIVPFFALNTQELKEVFAPSCLSCFDYVNGLADLVVGYMGAP----- --GWQWLVARNETGLEMLELARQCGLEEGPVDACGDRRAAVQQSITAYDRA--- --LTLPRWIAEFLAIIIGKIGPKGLEYARFSIDSHFTRNYLYVRRR- YPEKLDAHVPEYAKRIVSQYKLPDT-------
XP_005706147.1 coenzyme F420 hydrogenase beta subunit [*Galdieria sulphuraria*]	LGGKYDTRQYPAKSLCSHCGLCDTRFIHYVKDSCAFLN--- QHISELEYTVHGKSRDLEVENELYFGVHL- NMIAARRKQPLPGAQWSGIVTSIATRLLETGKVQGIVCVRNDEQDRFQPKPVL ATTPEEIYASRVNKPTLSPNLSVLDTVEASGFTRIGVIGVGCQVEALRSVQSKL GLEKLYVLGTPCVDNVTRKGLDKFLRTTSTSPDTVVHYEFMQDFRVHFKHDD GGPGKQWTETVPFFGLKTNELKDIFAPSCLSCFDYVNSLADLVVGYMGAP----- --GWQWIVVRNEIGMEMLDLVSS--IETMPLSSSGNRLQAVQNSIPAYDKG----- MTLPMWIAQLLGVVIDKLGPKGLEYARFSIDSHFTRNYLYMKRN- FPNILQRHVPEYARRIIEQYKLPKE-------

4.2 Strains and culture conditions

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

4.3 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). The pigments were separated through a Symmetry C8 column (4.6×150 mm, Waters) (Zapata, Rodríguez, and Garrido 2000). Detected peaks of each pigment at 440 nm were identified by their retention time and absorption spectrum.

4.4 Cloning of CBR, HCAR, and BciB from *Acaryochloris* RCC1774 and *Prochlorothrix hollandica*

Each coding region of CBR, HCAR, and BciB derived from *Acaryochloris* RCC1774 (WP_110986784.1, PZD72038.1, and PZD72398.1) and *Prochlorothrix hollandica* (WP_081599361.1, WP_017711629.1, and WP_044076442.1) were amplified by polymerase chain reaction from the genomic DNA of each species. The primers used for amplification are shown in Table 3. Amplified genes are cloned into pET-30a (+) vectors (Novagen) using the NdeI and XhoI sites through an in-fusion cloning system (Clontech).

Table 3. The list of primer for cloning each protein

	Forward	Reverse
AcaCBR	AAGGAGATATACATATGGCTGATTTATT TCCACTG	GGTGGTGGTGCTCGATCCGTGATCGCT TTATCCAGC
ProCBR	AAGGAGATATACATATGTCTATCCCTAT GGTTGTC	GGTGGTGGTGCTCGATGGCTTGGGCA GAGAAGGCCC
AcaHCAR	AAGGAGATATACATATGAGCATGACTG ATGATTGG	GGTGGTGGTGCTCGATCCGAAGCAGC CGCGAAATTT
ProHCAR	AAGGAGATATACATATGGGGGATCCGT TGCCAGGGGA	GGTGGTGGTGCTCGAGCCCCAGCAAT TGGCGAATTT
AcaFDVR	AAGGAGATATACATATgGCTAAATTCAT GACTGTT	GGTGGTGGTGCTCGATATCCGGTAGGG TGTACTGCT
ProFDVR	AAGGAGATATACATATGACCCAAGTTC CCTCGGTATCT	GGTGGTGGTGCTCGAGTTCCTTGGGC AATTGATACT

4.5 Expression and detection of recombinant proteins

The constructed plasmids for protein expression were introduced into *E. coli* (BL21). *E. coli* was grown and recombinant protein was expressed in an auto-induction medium (Studier 2005) at 18℃ 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 detect 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) and Western Lighting Plus-ECL (PerkinElmer Life Science), proteins were detected by fluorescence imaging.

4.6 Preparation of the chlorophyll derivatives

Divinyl chlorophyll was prepared from a *Synechocystis* mutant that lacks slr1923 (Ito et al. 2008). 7-Hydroxymethyl chlorophyll a was prepared through the reduction of chlorophyll b using NaBH4 according to a previous report (Holt 1959).

4.7 Enzyme assay

E. coli lysate (50 µl) prepared as described above was used for 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), 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℃ 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.

5. Reference

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