Distribution and functional analysis of the two types of 8-vinyl reductase involved in chlorophyll biosynthesis in marine cyanobacteria

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Abstract
In the chlorophyll biosynthesis pathway, the 8-vinyl group of the chlorophyll precursor is reduced to an ethyl group by 8-vinyl reductase. Two isozymes of 8-vinyl reductase have been described in oxygenic photosynthetic organisms: one encoded by *BciA* and another by *BciB*. Only BciB contains an [Fe-S] cluster and most cyanobacteria harbor this form; whereas a few contain *BciA*. Given this disparity in distribution, cyanobacterial BciA has remained largely overlooked, which has limited understanding of chlorophyll biosynthesis in these microorganisms. Here, we reveal that cyanobacterial *BciA* encodes a functional 8-vinyl reductase, as evidenced by measuring the *in vitro* activity of recombinant *Synechococcus* and *Acaryochloris* BciA. Genomic comparison revealed that *BciB* had been replaced by *BciA* during evolution of the marine cyanobacterium *Synechococcus*, and coincided with replacement of Fe-superoxide dismutase (SOD) with Ni-SOD. These findings imply that the acquisition of *BciA* confers an adaptive advantage to cyanobacteria living in low-iron oceanic environments.

Keywords
Synechococcus, *Acaryochloris*, 8-vinyl reductase, chlorophyll biosynthesis, adaptation, low-iron environment

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INTRODUCTION

Almost all the energy that sustains life on Earth is produced through photosynthesis. Chlorophyll is a pigment that captures light energy. All oxygenic photosynthetic organisms contain chlorophyll *a*, which is synthesized from glutamate through 14 enzymatic reactions (Bryant et al. 2020). In addition to chlorophyll *a*, green algae, plants, and some cyanobacteria possess chlorophyll *b* (Fig. 1). In plants, both chlorophylls are degraded enzymatically (Kuai et al. 2018). The first step in chlorophyll *a* degradation is the extraction of the central magnesium atom by Mg-dechelatase, which is encoded by *Stay-Green*, to form pheophytin *a*. The phytyl group of pheophytin *a* is then removed to form pheophorbide *a*, whose ring is oxidatively opened by pheophorbide *a* oxygenase, and the molecule is transported from the chloroplast to the vacuole for further degradation. The first step in chlorophyll *b* degradation is reduction to 7-hydroxymethyl chlorophyll *a* and then chlorophyll *a*, whose further catabolism is outlined above (Kuai et al. 2018). However, little is known with regard to chlorophyll degradation in cyanobacteria. To the best of our knowledge, Mg-dechelatase or pheophorbide *a* oxygenase have not been identified in cyanobacteria, and it remains unclear whether chlorophyll is degraded to smaller molecules within cyanobacterial cells.

During chlorophyll synthesis, chlorophyllide with a vinyl group at position 8 is produced as a precursor (Bryant et al. 2020). This molecule is also known as divinyl chlorophyllide because it has two vinyl groups, at positions 3 and 8, respectively. The vinyl group at position 8 is reduced to an ethyl group by 8-vinyl (8V) reductase to form chlorophyllide, which is then esterified with phytyl pyrophosphate to give chlorophyll. In photosynthetic bacteria, photosynthesis-related genes form clusters, and the corresponding enzymes can be identified by sequentially disrupting the genes in the cluster and examining the accumulated intermediate pigments (Bollivar et al. 1994). *BciA*, the gene encoding 8V reductase, was first identified in a mutant of *Arabidopsis thaliana* (hereafter *Arabidopsis*) that accumulated 8V chlorophyll (Beale 2005; Nagata et al. 2005). A homolog, also known as DVR or N-DVR, was found in photosynthetic bacteria (Liu and Bryant 2011) and other *BciA* homologues were found in some cyanobacteria and photosynthetic bacteria. Eventually, 8V reductase was identified in the cyanobacterium *Synechocystis* PCC6803 (hereafter *Synechocystis*) (Ito et al. 2008), where it was encoded by *BciB* (also known as F-DVR). This enzyme is not homologous to BciA. BciA contains no cofactor and its reductant is NADPH; whereas BciB contains an [Fe-S] cluster, has FAD as a cofactor, and uses ferredoxin (Fd) as a reductant (Table 1).

Land plants use BciA to synthesize chlorophyll, making BciB unnecessary. However, the existence of a homologue of *BciB*, 7-hydroxymethyl chlorophyll *a* reductase, which is involved in chlorophyll *b* conversion to chlorophyll *a* (Fig. 1) (Meguro et al. 2011), suggests that the plant lineage acquired BciA as a new 8V reductase. During this process, BciB, which remained as an 8V reductase for chlorophyll biosynthesis in cyanobacteria, was repurposed for chlorophyll *b* conversion.

Whereas cyanobacterial BciB has already undergone biochemical characterization (Ito et al. 2008; Lim et al. 2019), little is known about BciA, resulting in a limited understanding of chlorophyll biosynthesis in these microorganisms. In the present study, we examined the enzymatic activity of cyanobacterial BciA, as well as the relationship between *BciA* occurrence and iron availability in cyanobacteria habitats. The enzymatic activity of BciA and BciB recombinant proteins was determined and the reason for the existence of both genes in the cyanobacterium *Acaryochloris*

marina (hereafter *Acaryochloris*) is discussed. This study provides a better understanding of the enzymatic diversity supporting chlorophyll biosynthesis in cyanobacteria.

MATERIALS AND METHODS

Materials and growth conditions

For genome analysis, we used representative marine cyanobacteria (Scanlan et al. 2009), whose entire genome sequences were registered with the Kyoto Encyclopedia of Genes and Genomes (KEGG), from which their 16S rRNA nucleotide sequence was obtained. The 16S rRNA nucleotide sequences of *Synechococcus* WH5701, *Synechococcus* WH7805, and *Synechococcus* RS9916 were obtained from the National Center for Biotechnology Information (NCBI). *Synechocystis* was grown at 24 °C in BG11 medium under continuous illumination (50 μmol photons m^{-2} s^{-1}).

Sequence analysis and phylogenetic analysis

A search was performed using KEGG and NCBI databases. Accession numbers are listed in Table 2. In phylogenetic analysis, MEGA 10 software (Kumar et al. 2018) was used for sequence alignment and the construction of neighbor-joining trees.

Enzymatic assay of recombinant 8V reductase

Synechocystis BciB, encoded by *slr1923*, was cloned into pET-30a (+) vectors (Novagen), as previously reported (Ito and Tanaka 2014). *Synechococcus* WH8102 (CCMP2370) was obtained from the National Center for Marine Algae and Microbiota, USA. *BciA* (*synw0963*) was amplified from *Synechococcus* WH8102 genomic DNA using the primer sets 5- CAT ATG GCT CTG CGT CAC GAC 3' and 5- CTC GAG GAA CAA AGC TGC ATC -3' (the underlined sections are engineered *Nde*I and *Xho*I sites, respectively). The amplified DNA fragments were cloned into pET-30a (+) vectors as previously reported (Nagata et al. 2005). *Acaryochloris marina* MBIC11017 was obtained from the National Institute of Technology and Evolution, Japan. *Acaryochloris BciA* (AM1_2394) and *BciB* (AM1_2849) were amplified from *Acaryochloris* genomic DNA using the primer sets 5′- AAG GAG ATA TAC ATA TGA CTG ACG CTA GCA CTT CG -3′ and 5′- GGT GGT GGT GCT CGA TGA ATA CAG CGA AGT C-3′, and 5′- AAG GAG ATA TAC ATA TGA CTG CGG TAC AAC CCC AT -3′ and 5′- GGT GGT GGT GCT CGA TGT CCG GTA AGT CAT ATT GAC -3′, respectively. The amplified DNA fragments were cloned into pET-30a (+) vectors, as previously reported (Lim et al. 2019). The recombinant protein was expressed in *Escherichia coli* in auto-induction medium at 37 °C overnight (Studier 2005). A 1-mL aliquot of the cell suspension was harvested by centrifugation at 20,000 × *g* for 1 min. The pellet was resuspended in 100 μL BugBuster Protein Extraction Reagent (Merck). For BciA analysis, 1 μL of 50 mM NADPH was added. For BciB analysis, 1 μL spinach ferredoxin-$NADP^+$ reductase (FNR; 0.1 mg mL^{-1}, Sigma-Aldrich), 1 μL spinach Fd (1 mg mL^{-1}, Sigma-Aldrich), and 1 μL of 50 mM NADPH were added to 50 μL of the *E. coli* lysate. Pigments were prepared as previously reported (Ito and Tanaka 2014), solubilized in DMSO, and 0.5 μL of the solution (500 pmol of pigments) was used. The mixture was incubated at 25 °C for 30 min, and the reaction was stopped by adding 200 μL acetone. After centrifugation at 20,000 × *g* for 10 min, the supernatant was subjected to high-performance liquid chromatography (HPLC) using a diode array detector (SPD-M10A, Shimadzu) as previously reported (Lim et al. 2019). For reduction analysis of chlorophyllide *b* by *Synechocystis* BciB, Fd, FNR, and NADPH were added five times, the solution was incubated for 30 min, the pigments were mixed in, and the reaction was allowed to proceed for a further 90 min.

Transformation of *Arabidopsis* and *Synechocystis*

To construct 8V reductase-deficient *Synechocystis*, the *slr1923* locus was disrupted with a chloramphenicol resistance cassette, as reported previously (Ito et al. 2008). Transformants were selected on solid BG-11 medium containing 25 μg mL^{-1} chloramphenicol. *Arabidopsis BciA* (AT5G18660) cDNA was amplified with primers 5′- GAT ATC ATG GCT CTG CGT CAC GAC TT-3′ and 5′- GAT ATC TCA GAA CAA AGC TGC ATC GC -3′, and introduced into *Synechocystis* under the *psbA2* promoter using the kanamycin resistance cassette for selection (Ito and Tanaka 2011). Transformants were selected on solid BG-11 medium containing 25 μg mL^{-1} kanamycin. *Synechocystis BciB* was introduced into a *BciA*-deficient *Arabidopsis* (Nagata et al. 2005) with chlorophyllide *a* oxygenase transit peptide. *Synechocystis BciB* and *Arabidopsis* chlorophyllide *a* transit peptide were amplified using the primer sets 5′- ATG ACC GTT CCT GCC CCC CA -3′ and 5′- GAG CTC TTA TTG CTG GGG AAG TTT AT -3′ (the underlined section is an engineered *Sac*I site), and 5′- TCT AGA ATG AAC GCC GCC GTG TTT AG -3′ and 5′- TGG GGG GCA GGA ACG GTC ATA GGT CTC CAT TGA CTC TTC T -3′ (the underlined section is an engineered *Xba*I site), respectively. The obtained DNA fragments were mixed and further amplified using primers 5′- TCT AGA ATG AAC GCC GCC GTG TTT AG -3′ and 5′- GAG CTC TTA TTG CTG GGG AAG TTT AT -3′. The generated DNA fragments were introduced in the pGreenII vector (Hellens et al. 2000) under the 35S promoter using the *Xba*I and *Sac*I sites. The *BciA*-deficient *Arabidopsis* mutant was transformed with the resulting plasmids. Transformants were selected based on their hygromycin resistance as reported previously (Nagata et al. 2005).

Pigment analysis of *Synechocystis* and *Arabidopsis*

Synechocystis cells were harvested by centrifugation, and pigments were extracted with methanol, as reported previously (Ito et al. 2008). Pigments of *Arabidopsis* leaves were extracted with acetone and analyzed by HPLC, as reported previously (Meguro et al. 2011).

Immunoblot analysis of *Synechocystis* and *Arabidopsis*

A 1-mL aliquot of *Synechocystis* culture harvested at OD_{750} = 5 was centrifuged at 20,000 × *g* for 1 min and the supernatant was discarded. The pellet was resuspended in 0.1 mL water. The cells were lysed five times with glass beads (0.1 mm diameter) at 5000 rpm for 30 s using a Mini-bead beater. The homogenate was mixed with the same volume of sample buffer containing 125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% (w/v) sucrose, and 5% (v/v) 2-mercaptoethanol. *Arabidopsis* leaf disks (0.25 cm^2) were frozen in liquid nitrogen, macerated with a pestle in a microtube, and resuspended in sample buffer. Homogenates of *Synechocystis* and *Arabidopsis* were incubated at 90 °C for 1 min. After centrifugation at 20,000 × *g* for 3 min, 10 μL of the *Synechocystis* supernatant or 15 μL of the *Arabidopsis* supernatant was subjected to SDS-PAGE, and the proteins were transferred onto a polyvinylidene difluoride membrane for immunoblotting (Lim et al. 2019). Anti-*Synechocystis* BciB and anti-*Arabidopsis* BciA antisera were obtained from rabbits immunized with *Synechocystis* BciB and *Arabidopsis* BciA, which had been purified with a nickel column (Ito and Tanaka 2014). Primary antibodies were diluted with an immunoreaction enhancer solution (Can Get Signal, Toyobo). Anti-rabbit IgG linked to horseradish peroxidase was used as the secondary antibody. Horseradish peroxidase activity was visualized using a Western Lightning Plus-ECL Chemiluminescence Detection Kit (PerkinElmer).

RESULTS

Enzymatic activity of 8V reductase encoded by *BciA*

The phylogenetic tree of cyanobacterial BciB was almost identical to that obtained by 16S rRNA. In contrast, the phylogenetic tree of BciA was markedly different, suggesting that *BciA* had been laterally transferred to cyanobacteria (Chen et al. 2016). Cyanobacterial *BciA* has been reported to

comprise two clades: one formed by marine *Synechococcus*, and the other by *Acaryochloris* and *Leptolyngbya*. *Acaryochloris* BciA and BciB activities were tested by complementation in *BciB*-deficient *Synechocystis* (Chen et al. 2016). However, BciA activity in none of the two clades had been determined biochemically. Here, we examined the enzymatic activity of recombinant BciA derived from *Synechococcus* WH8102 and *Acaryochloris*. After incubation of 8V chlorophyllide with these recombinant proteins, a chlorophyllide with an ethyl group at position 8 was detected (Fig. 2). These results confirm that *BciA* from marine cyanobacteria encodes 8V reductase.

Distribution of 8V reductase in marine cyanobacteria

Considering marine cyanobacteria are the most abundant photosynthetic organisms on Earth (Scanlan et al. 2009), the ecology of their genomes has been extensively studied. Marine cyanobacteria are clustered into sub-clusters 5.1A, 5.1B, 5.2, and 5.3, based on 16S rRNA gene sequences. All of the sub-clusters are formed by *Synechococcus*; whereas *Prochlorococcus* forms a cluster of its own. The distribution of *BciA* and *BciB* revealed that *Synechococcus* harboring *BciB* belonged to sub-clusters 5.2, 5.3, and 5.1B; whereas *Synechococcus* harboring *BciA* belonged to sub-cluster 5.1A (Fig. 3). Because all freshwater cyanobacteria possess *BciB*, the phylogenetic tree indicated that marine *Synechococcus* most likely developed from freshwater cyanobacteria and *BciA* was acquired in the process of forming sub-cluster 5.1A. Considering that freshwater contains more iron than seawater, the predominance of sub-cluster 5.1A cyanobacteria in the ocean (Zwirglmaier et al. 2008) may reflect adaptation to a low-iron environment.

Comparison of the distribution of 8V reductase and superoxide dismutase (SOD)

Given that marine cyanobacteria are found in oligotrophic environments, their relationship with environmental nutrients has been extensively studied (Scanlan et al. 2009). Iron is particularly limiting in oceans. SOD, which scavenges reactive oxygen species, contains iron, copper-zinc, or nickel in its reactive site. Ni-SOD is thought to enable adaptation to iron-poor environments. *Fe-SOD* and *Ni-SOD* show mutually exclusive distributions in marine cyanobacteria (Dupont et al. 2008). Here, a comparison of their distributions with that of 8V reductase revealed that *BciB* occurred in conjunction with *Fe-SOD* and *BciA* with *Ni-SOD* (Fig. 3). Co-distribution of *BciA* with *Ni-SOD* in marine *Synechococcus* reinforces the idea that acquisition of *BciA* represents an adaptation to iron-poor environments.

Loss of *BciB* in the progenitor of *Prochlorococcus*

Prochlorococcus inhabits the oligotrophic region of oceans (Partensky and Garczarek 2010) and is grouped closely with oceanic cyanobacteria based on 16S rRNA (Scanlan et al. 2009). Our phylogenetic tree indicates that the 8V reductase lost by *Prochlorococcus* was encoded by *BciB* not *BciA* (Fig. 3). The observation implies that it was advantageous for *Prochlorococcus* to lose iron-demanding *BciB* for adaptation to iron-poor environments, under which demand for iron by BciB would have been a disadvantage.

BciB of *Synechocystis* PCC6803, a model cyanobacterium without chlorophyll *b*, exhibits both 7-hydroxymethyl chlorophyll *a* reductase and 8V reductase activity. Furthermore, *Synechocystis* BciB was suggested to mediate the interconversion between 7-hydroxymethyl chlorophyll *a* and chlorophyll *b* (Ito and Tanaka 2014). These multiple activities were not expected because reduction/oxidation of the hydroxy group/hydroxymethyl group and reduction of the hydroxymethyl group to the methyl group represent different reactions. To confirm that chlorophyll *a* was generated from chlorophyll *b* by BciB, we examined the pigments produced. Chlorophyllide *b* was used as substrate instead of chlorophyll due to its greater aqueous solubility, and was incubated with recombinant *Synechocystis* BciB. Pigment analysis by HPLC revealed a peak at the position of

chlorophyllide *a* (Fig. 4a). The peak's identity was confirmed by an absorption spectrum (Fig. 4b). These results show that *Synechocystis* BciB can convert chlorophyll *b* to chlorophyll *a*. In *Synechocystis*, such activity has no impact on its metabolism, since they do not synthesize chlorophyll *b*. Conversely, *Prochlorococcus* acquired a capacity to synthesize a chlorophyll *b* derivative (a 8-vinyl form of chlorophyll *b*). Therefore, the chlorophyll *b*-to-*a* conversion activity of BciB, if it occurs in a cell, would disrupt the balance between chlorophyll *a* and chlorophyll *b* in *Prochlorococcus.* Therefore, the prevention of unnecessary chlorophyll *b*-to-*a* conversion is a potential alternative reason for the BciB loss in *Prochlorococcus*.

Interchange between BciA and BciB

Plants harbor a large multi-enzyme complex responsible for chlorophyll biosynthesis (Winkel 2004). Within this complex, biosynthetic intermediates are transferred between catalytic sites without the need for diffusion. Accordingly, glutamyl-tRNA reductase and glutamate semialdehyde aminomutase enable the transfer of relatively unstable intermediates from one active site to the next. If 8V reductase formed a complex with the enzymes responsible for the preceding or following metabolic steps, it would be unfavorable to replace BciB with BciA or *vice versa*. To examine the feasibility of exchanging 8V reductase, we replaced the cyanobacterial and plant 8V reductases. *BciB*-deficient *Synechocystis* accumulated 8V chlorophyll. When *Arabidopsis BciA* was introduced into this mutant, chlorophyll with an ethyl group at position 8 was detected (Fig. 5a). Accumulation of the introduced 8V reductase was confirmed by immunoblotting, indicating that *BciA* complemented the loss of *BciB*. Similarly, when *Synechocystis BciB* was introduced into *BciA*-deficient *Arabidopsis*, the accumulation of 8-ethyl chlorophyll suggested that *BciA* was successfully complemented by *BciB* (Fig. 5b). These results indicate that BciA and BciB can be readily exchanged, and *BciA* can be efficiently transferred to cyanobacteria.

Genome map of 8V reductase and SOD

So far, *Prochlorococcus* was thought to have lost *BciA* during evolution (Nagata et al. 2005). However, our 16S rRNA phylogenetic tree indicates that *Prochlorococcus* lost *BciB* instead (Fig. 3). A reexamination of the *BciA* and *BciB* loci in the cyanobacterial genome revealed that *BciA* was inserted in place of *BciB* (Fig. 6). Because BciA and BciB share no nucleotide sequence similarity, *BciA* should not be inserted into the *BciB* locus by homologous recombination. Similarly, neither Fe-SOD and Ni-SOD nucleotide sequences were homologous, yet Fe-SOD of *Synechococcus* WH7803 and Ni-SOD of *Synechococcus* WH8102 shared the same locus (Fig. 6). Hence, while the underlying recombination mechanism needs to be identified, it may not be unusual for functionally identical genes without homology to occupy the same locus.

Activity of *Acaryochloris* BciA and BciB

The physiological determinant driving the insertion of *BciA* in place of *BciB* in cyanobacteria remains unknown. The fact that both *BciA* and *BciB* are present at the same genomic locus disputes the insertion of *BciA* at an arbitrary position in the genome and subsequent loss of *BciB*. Instead, these cyanobacteria always possessed only one 8V reductase. One possible reason is that having two types of 8V reductase poses a disadvantage. However, the genome of *Acaryochloris* harbors genes homologous to both *BciA* and *BciB*. BciA was found to catalyze the reduction of the 8 vinyl group (Fig. 2); whereas recombinant BciB yielded 8-ethyl chlorophyllide (Fig. 2). These findings suggest that both *BciA* and *BciB* encode active 8V reductases and their coexistence is not problematic for *Acaryochloris*.

DISCUSSION

Enzyme diversity in the chlorophyll biosynthetic pathway

In the chlorophyll biosynthetic pathway, isozymes are found in one species (Bryant et al. 2020). A typical example is the reduction of protochlorophyllide to chlorophyllide. In the light, the reaction is catalyzed by an NADPH-dependent protochlorophyllide oxidoreductase; in the dark, it is catalyzed by a nitrogenase-like protochlorophyllide oxidoreductase. Cyanobacteria possess also two types of coprophyrinogen III oxidase and Mg-protoporphyrin IX monomethylester cyclase to be used under aerobic or anaerobic conditions (Fujita et al. 2015). Three different types of protoporphyrinogen IX oxidase (PPOX) exist among cyanobacteria (Kobayashi et al. 2014). With the exception of *Acaryochloris*, cyanobacteria present an 8V reductase encoded by either *BciA* or *BciB*. In the present study, *BciA* of marine *Synechococcus* belonging to sub-cluster 5.1A and *Acaryochloris* exhibited 8V reductase activity. This is the first study to report enzymatic activity of cyanobacterial BciA. In some photosynthetic bacteria, reduction of the vinyl group at position 8 is achieved by chlorophyllide *a* oxidoreductase, a distinct enzyme from BciA and BciB (Harada et al. 2014; Yamamoto et al. 2020), implying great diversity among 8V reductases.

At the time when life emerged on Earth, the oceans were characterized by reducing conditions without dissolved molecular oxygen. As cyanobacteria carried out oxygen-evolving photosynthesis, oxygen became more abundant, causing iron to oxidize and sediment, eventually lowering the amount of dissolved iron (Saito et al. 2003). Because cyanobacteria require a certain amount of iron, they are believed to have evolved ways to cope with iron-deficient conditions. *Prochlorococcus* is an abundant photosynthetic organism in low-latitude iron-poor waters. It adapts to extremely iron-poor environments by reducing the amount of iron-rich photosystem I (Partensky and Garczarek 2010). Diatoms, which are abundant at mid- to high-latitudes, adapt to iron-poor environments by reducing photosystem I and using ferritin to store iron (Armbrust 2009). Adaptation to low-iron conditions is indispensable for ocean dominance. The diversity of genes involved in chlorophyll biosynthesis in cyanobacteria has been linked to light and oxygen availability (Fujita et al. 2015). In this study, environmental iron was also shown to affect the diversity of enzymes involved in chlorophyll biosynthesis.

Characteristics of chlorophyll in *Prochlorococcus*

Prochlorococcus chlorophylls are characterized by the presence of a vinyl group at position 8 and chlorophyll *b*-type chlorophylls. *Prochlorococcus* is thought to have lost its 8V reductase to accumulate 8V chlorophyll and thus absorb blue light more efficiently (Ting et al. 2002). The Soret band of chlorophyll *a* shifts to a longer wavelength when the ethyl group is replaced by a vinyl group at position 8. Because at that wavelength, the absorption spectra of 8V chlorophyll *a* and chlorophyll *b* overlap (Shedbalkar and Rebeiz 1992), there is little advantage in shifting the Soret band of chlorophyll *a* to a longer wavelength if chlorophyll *b* is also present. The absence of 8V chlorophyll *a*-possessing cyanobacteria devoid of a chlorophyll *b* moiety suggests that the red-shifted Soret band of chlorophyll *a* does not confer a significant advantage to cyanobacteria. This means that the only advantage of possessing 8V chlorophyll comes from the red shift of the Soret band of chlorophyll *b*. Considering BciB has the potential to convert chlorophyll *b* into chlorophyll *a*, loss of BciB was potentially necessary to maintain chlorophyll *b* levels in *Prochlorococcus* progenitors once it acquired the capacity to synthesize chlorophyll *b*. Although the D1 protein in the reaction center of photosystem II required optimization to enable the use of 8V chlorophyll (Ito and Tanaka 2011), this cost might have been compensated by the acquisition of chlorophyll *b*. The potential of BciB to convert chlorophyll *b* could have influenced the evolution of *Prochlorococcus*. In summary, the primary reason for the loss of 8V reductase is to shift the Soret band to a longer wavelength; however, loss of 8V reductase may have also favored adaptation to a low-iron environment and accumulation of chlorophyll *b*.

Insertion position of horizontally transferred genes

Although *BciA* and *BciB* do not possess homologous nucleotide sequences and cannot be exchanged by homologous recombination, they occupy the same genomic locus. PPOX and SOD constitute a similar example (Dupont et al. 2008; Kobayashi et al. 2014). The reason why *BciA* and *BciB* share the same locus remains to be determined. If the coexistence of two isozymes of 8V reductases was detrimental for the cell, insertion of *BciA* in place of *BciB* could be thought of as providing more stability. Except for *Acaryochloris*, it is unlikely that two isozymes of 8V reductase are present simultaneously. If *BciB* expression requires tight regulation, *BciA* may be inserted in place of *BciB* to use the same promoter. Nevertheless, there are presently no reports that *BciB* expression is tightly controlled. In addition, in plants, *BciA* does not show significant variation in expression, and 8V reduction is not considered a major regulatory step in the chlorophyll biosynthetic pathway. Therefore, expression regulation may not be the reason for sharing the same 8V reductase locus. *Synechocystis BciB* and *Arabidopsis BciA* could successfully complement each other (Fig. 5). Similarly, PPOX encoded by *HemY* can be complemented by PPOX encoded by *HemJ* (Kato et al. 2010), and both genes could be found in the same locus. The results suggest that enzymes encoded by non-homologous genes but present at the same locus are easily replaceable. This functional compatibility may be explained by the fact that insertion at an arbitrary position could disrupt a gene at the locus, which would be disadvantageous to cyanobacteria.

Diverse distribution of *BciA* and *BciB* in cyanobacteria

BciA and *BciB* are mutually exclusive in cyanobacteria; only in *Acaryochloris* are both genes present. We hypothesize two reasons why *Acaryochloris* possesses both *BciA* and *BciB*. First, the two genes are expressed differentially depending on iron availability: BciB requires more iron than BciA and is not suited to low-iron conditions (Chen et al. 2016). However, we could not find a significant difference in gene expression between *BciA* and *BciB* under low-iron conditions (data not shown). The second is functional differentiation: BciA is involved in chlorophyll synthesis, whereas BciB is involved in chlorophyll *d* degradation. In the case of land plants, when chlorophyll *b* is degraded, the formyl group at position 7 is reduced to a methyl group. This is because the chlorophyll moiety with the formyl group at the 7-position cannot be a substrate for pheophorbide *a* oxygenase (Hörtensteiner et al. 1995). If we assume that chlorophyll *d* is degraded by an enzyme similar to plant pheophorbide *a* oxygenase, the formyl group of chlorophyll *d* could be reduced to a methyl group with a hydroxymethyl as an intermediate. We hypothesized that *Acaryochloris* BciB could be involved in the conversion of a hydroxymethyl chlorophyll derivative to a methyl derivative. Recombinant BciB was incubated with 3-hydroxymethyl chlorophyll *a*, which is the predicted intermediate in the chlorophyll *d* degradation pathway. HPLC analysis of the resulting pigments did not produce any peak at the time when a methyl group at position 3 was expected (data not shown). Hence, we could not show that *Acaryochloris* BciB was involved in chlorophyll degradation. The fact that both *BciA* and *BciB* are induced under increased chlorophyll biosynthesis (Yoneda et al. 2016) confirms the role of BciB in the biosynthetic pathway; however, the physiological significance of having both BciA and BciB remains unknown.

Compared to BciB, which has an [Fe-S] cluster and FAD, BciA has a simpler structure (Table 1). In addition, as there are no examples of BciA being less active than BciB, the former appears to offer more advantages than the latter. *Acaryochloris* could be in a transitional state, whereby 8V reductase is undergoing a shift from BciB to BciA. The NCBI database shows that cyanobacterial symbionts found in marine sponges, such as *Candidatus* Synechococcus spongiarum SP3, possess a form of *BciA*, which is highly homologous to *Acaryochloris BciA*. Symbiotic strains of

Acaryochloris have also been observed (Ohkubo and Miyashita 2012), suggesting that BciA may, for unknown reasons, favor adaptation in symbiotic situations.
Land plants use BciA as the 8V reductase in chlorophyll synthesis and 7-hydroxymethyl chlorophyll *a* reductase, which is homologous to BciB, in the conversion of chlorophyll *b*. *Acaryochloris* RCC1774 possesses chlorophyll *b* instead of chlorophyll *d* (Partensky et al. 2018). *Acaryochloris* RCC1774 and *Prochlorothrix hollandica*, both of which have chlorophyll *b*, use BciB as the 8V reductase and 7-hydroxymethyl chlorophyll *a* reductase for the conversion of chlorophyll *b* (Lim et al. 2019). To the best of our knowledge, these are the only cyanobacterial species that possess both BciB and 7-hydroxymethyl chlorophyll *a* reductase. The diversity of 8V reductase suggests that this enzyme may have played an important role in the evolution of chlorophyll metabolism.
Prochlorococcus, which has both chlorophyll *a* and chlorophyll *b* with a vinyl group at position 8, is abundant in oligotrophic oceanic waters; however, the same cannot be said of cyanobacteria possessing only chlorophyll *a* with a vinyl group at position 8. The presence of both chlorophyll *a* and chlorophyll *b*, as found in green algae, would be advantageous in photosynthesis, but such cyanobacterial species are few and are not dominant. The characteristics of 8V reductase may be responsible for this unexpected distribution of cyanobacterial chlorophyll. Understanding the role of 8V reductase in adaptation to the environment will shed light on the diversity of cyanobacteria.

CONCLUSION

In this study, we employed biochemical analysis to demonstrate that BciA of marine cyanobacteria catalyzed 8-vinyl group reduction during chlorophyll biosynthesis. Both marine cyanobacteria and land plants possess BciA. However, they may have acquired it for different reasons: marine cyanobacteria have acquired BciA to adapt to low-iron environments; land plants to direct BciB towards chlorophyll *b* degradation. *Acaryochloris* is somewhere in between and might be undergoing adaptation to a low-iron environment. The 8V reductase may have played an important role in the development of chlorophyll metabolism.

FIGURE CAPTIONS

Fig. 1 Chlorophyll metabolic pathway.
The dashed circle indicates the reduction/oxidation site. 8V reductase is encoded by *BciA* or *BciB*. Chl syn, chlorophyll synthase; CAO, chlorophyllide *a* oxygenase; CBR, chlorophyll *b* reductase; HCAR, 7-hydroxymethyl chlorophyll *a* reductase

Fig. 2 Enzymatic analysis of marine *Synechococcus* and *Acaryochloris* 8V reductase.
BciA of *Synechococcus* WH8102, as well as BciA and BciB of *Acaryochloris* were expressed in *E. coli*. The *E. coli* lysate was incubated with 8V chlorophyllide *a* at 25 °C for 30 min. Pigments were extracted and analyzed by HPLC. The peaks detected for each pigment were identified by their retention times. Chlide, chlorophyllide; 8102 BciA, *Synechococcus* WH8102 BciA; AmBciA, *Acaryochloris* BciA; AmBciB, *Acaryochloris* BciB

Fig. 3 Phylogenetic distribution of 8V reductase and SOD genes.
A phylogenetic tree of 8V reductase and SOD was constructed based on the 16S rRNA genome of marine cyanobacteria. The 16S rRNA gene of *Synechocystis* was chosen as the out-group. Sub-clusters were labeled based on a previous report (Scanlan et al. 2009). Representative species were selected to construct a phylogenetic tree. Values at branch points indicate bootstrap support (1000 replicates). Bootstrap values of >60% are shown. The number of nucleotide substitutions per site is illustrated using a scale bar.

Fig. 4 Enzymatic analysis of *Synechocystis* BciB against chlorophyllide *b*.
(a) HPLC profiles of the pigments after incubation of chlorophyllide *b* with recombinant *Synechocystis* BciB. Chlorophyllide *b* was incubated with lysate obtained from *E. coli* expressing *Synechocystis* BciB with NADPH, FNR, and Fd. The arrow indicates the chlorophyllide *a* produced from chlorophyllide *b*. (b) HPLC absorption spectra of the chlorophyllide *a* standard and the peak highlighted in (a). Chlide, chlorophyllide

Fig. 5 Complementation of 8V reductase mutants with *BciA* or *BciB.*
(a) *Synechocystis* mutant analysis. The pigments were extracted from *Synechocystis* and analyzed by HPLC. Proteins were extracted from *Synechocystis* and 8V reductase was detected using specific antibodies. Wild-type, 8V reductase-deficient mutant, and mutants complemented with *Arabidopsis* BciA were examined. Because cyanobacteria contain multiple chromosome copies, gene disruption may be leaky, noted in the blot by weak bands. (b) *Arabidopsis* mutant analysis. The pigments were extracted from *Arabidopsis* and analyzed by HPLC. Proteins were extracted from *Arabidopsis* and 8V reductase was detected using specific antibodies. Wild-type, 8V reductase-deficient mutant, and mutants complemented with *Synechocystis* BciB were examined. The *Arabidopsis* BciA mutation created a single base pair change, which resulted in an amino acid substitution and the accumulation of low levels of mutant BciA, noted in the blot by weak bands. SyBciB, *Synechocystis* BciB; AtBciA, *Arabidopsis* BciA; WT, wild-type

Fig. 6 Synteny in the *BciA*/*BciB* and *Fe-SOD*/*Ni-SOD* replacement region in *Synechococcus* and *Prochlorococcus.*
Arrangement of genes in the regions containing *BciA*/*BciB* or *Fe-SOD*/*Ni-SOD* in representative species of *Synechococcus* and *Prochlorococcus* is shown.

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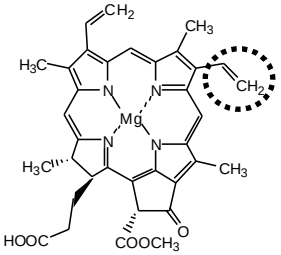
Table 1 BciA and BciB properties

	electron donor	coenzyme	Fe-S cluster
BciA	NADPH	-	-
BciB	Fd	FAD	+

Table 2 Accession numbers of 16S rRNA, BciA, and BciB

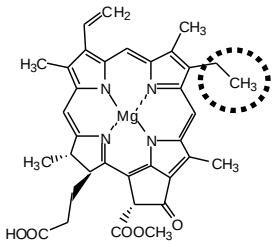
	16S rRNA	BciA	BciB
Prochlorococcus CCMP1378	RNA_39		
Prochlorococcus CCMP1375	Pro_r01		
Prochlorococcus MIT9303	P9303_rrs1309420		
Prochlorococcus MIT9301	P9301_rrs1309398		
Prochlorococcus MIT9313	PMT_RNA_46-3		
Synechococcus WH 8103	SynWH8103_02514	SynWH8103_01101	
Synechococcus WH 8109	Syncc8109_2421	Syncc8109_1014	
Synechococcus KORDI-52	KR52_14500	KR52_08165	
Synechococcus CC9605	syncc9605_R0051	Syncc9605_1613	
Synechococcus CC9902	syncc9902_R0046	Syncc9902_1374	
Synechococcus WH8102	RNA_53	synw0963	
Synechococcus RS9916	AY172826		WP_038023317
Synechococcus WH7805	AF001478		WP_006041718
Synechococcus WH7803	RNA_51		SynWH7803_1550
Synechococcus RCC307	RNA_8		SynRCC307_0774
Synechococcus WH5701	AY172832		EAQ74014
Synechocystis PCC6803	rrn16Sa		slr1923

Fig. 1



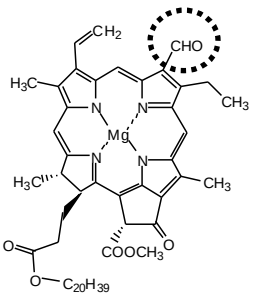
8-vinyl chlorophyllide *a*

8V reductase
(BciA/BciB)



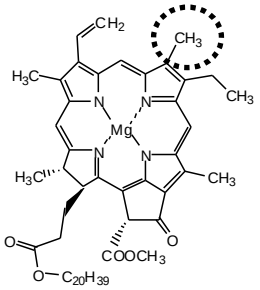
chlorophyllide *a*

Chl syn



chlorophyll *b*

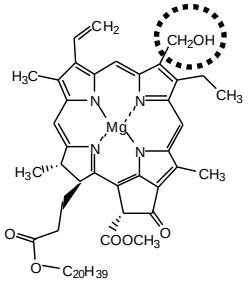
CAO



chlorophyll *a*

HCAR

CBR



7-hydroxymethyl
chlorophyll *a*

Fig. 2

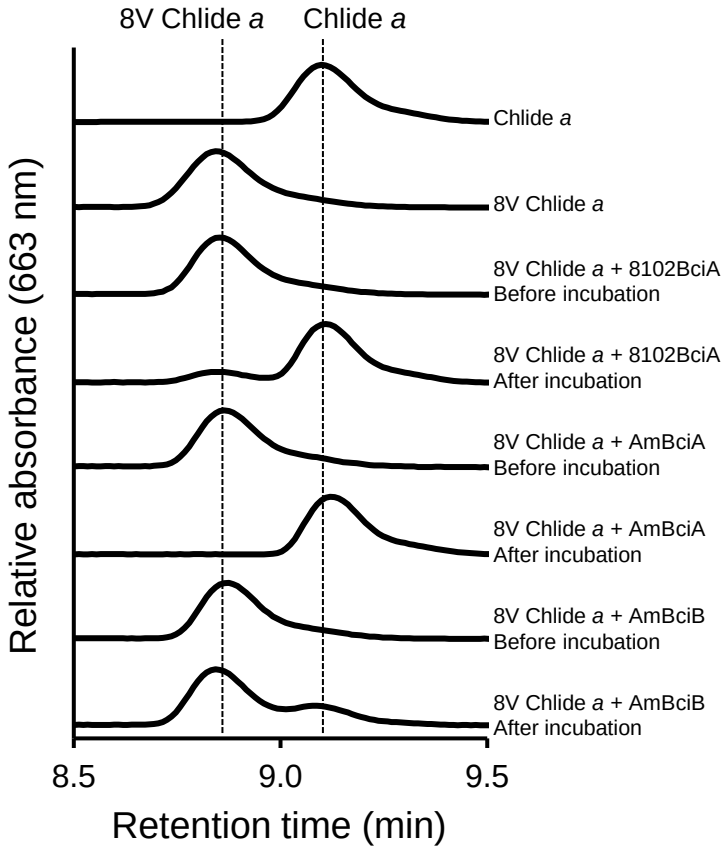
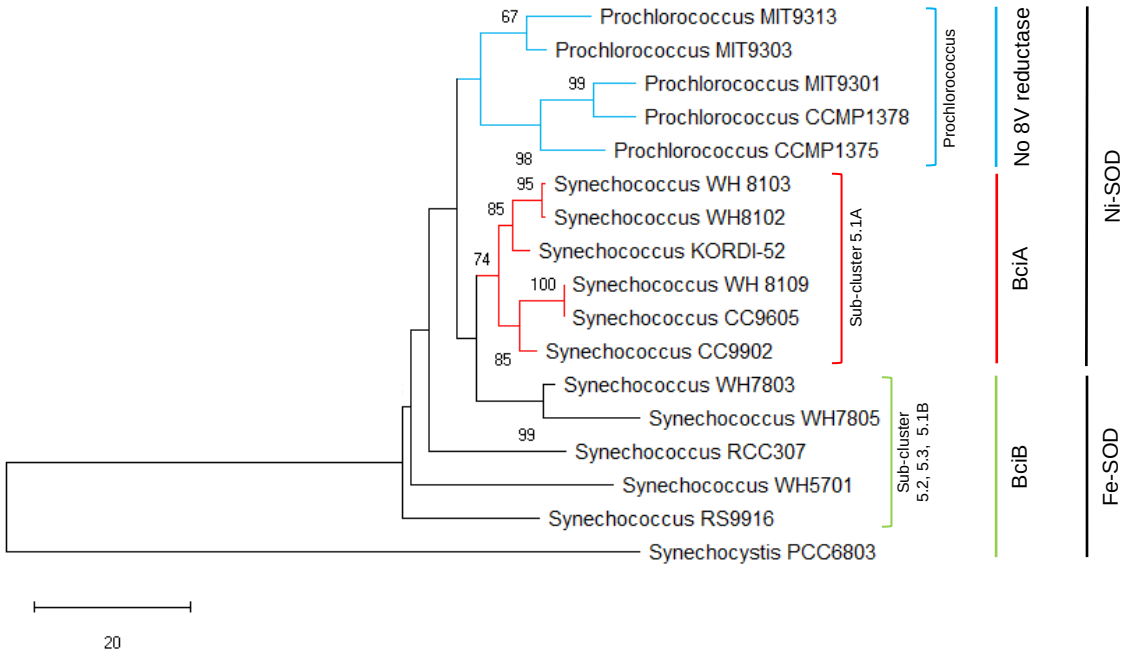

8V Chlide a
Chlide a
Chlide a
8V Chlide a
8V Chlide a + 8102BciA
Before incubation
8V Chlide a + 8102BciA
After incubation
8V Chlide a + AmBciA
Before incubation
8V Chlide a + AmBciA
After incubation
8V Chlide a + AmBciB
Before incubation
8V Chlide a + AmBciB
After incubation
Relative absorbance (663 nm)
8.5
9.0
9.5
Retention time (min)

Fig. 3


Prochlorococcus MIT9313
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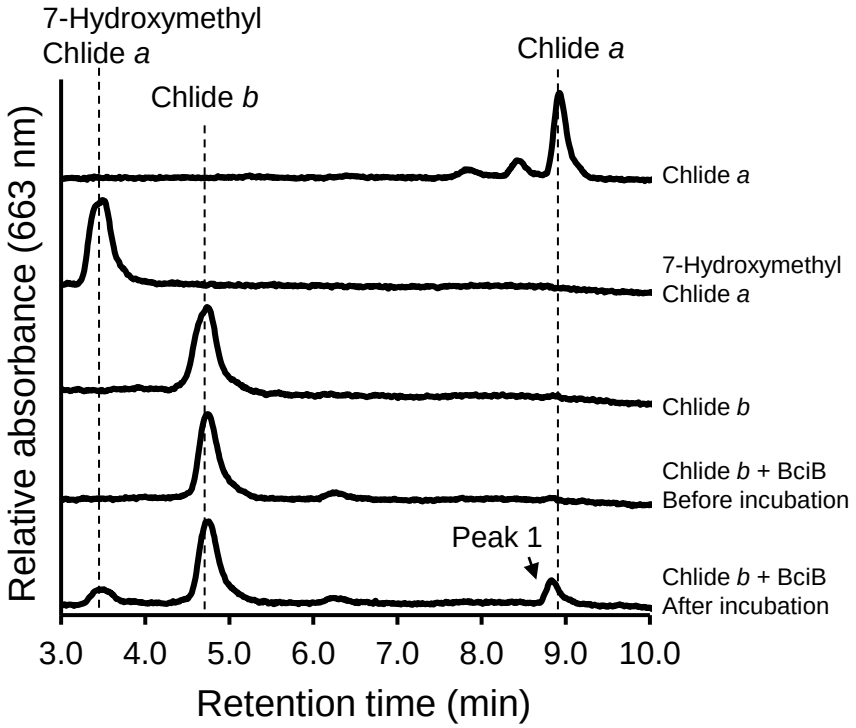
Prochlorococcus CCMP1378
Prochlorococcus CCMP1375
Synechococcus WH 8103
Synechococcus WH8102
Synechococcus KORDI-52
Synechococcus WH 8109
Synechococcus CC9605
Synechococcus CC9902
Synechococcus WH7803
Synechococcus WH7805
Synechococcus RCC307
Synechococcus WH5701
Synechococcus RS9916
Synechocystis PCC6803
67
99
98
95
85
74
100
85
99
Prochlorococcus
Sub-cluster 5.1A
Sub-cluster 5.2, 5.3, 5.1B
No 8V reductase
BciA
BciB
Ni-SOD
Fe-SOD
20

Fig. 4

(a)


7-Hydroxymethyl
Chlide a
Chlide b
Chlide a
Relative absorbance (663 nm)
Chlide a
7-Hydroxymethyl
Chlide a
Chlide b
Chlide b + BciB
Before incubation
Peak 1
Chlide b + BciB
After incubation
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Retention time (min)

(b)

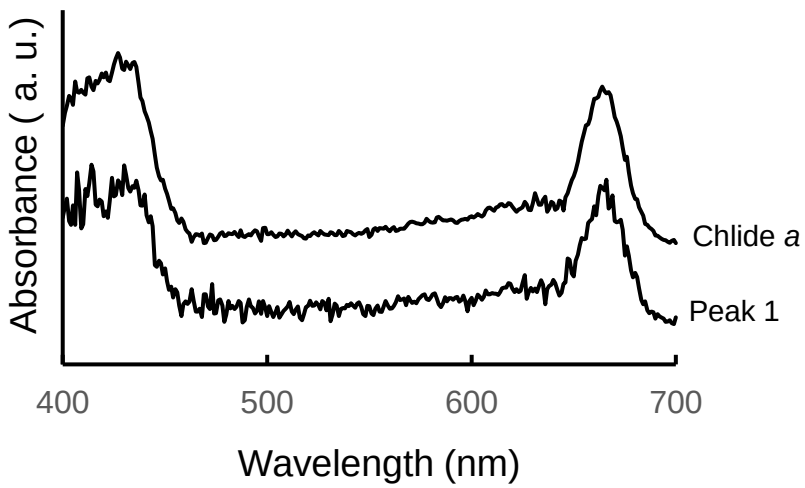

Absorbance (a. u.)
Chlide a
Peak 1
400
500
600
700
Wavelength (nm)

Fig. 5

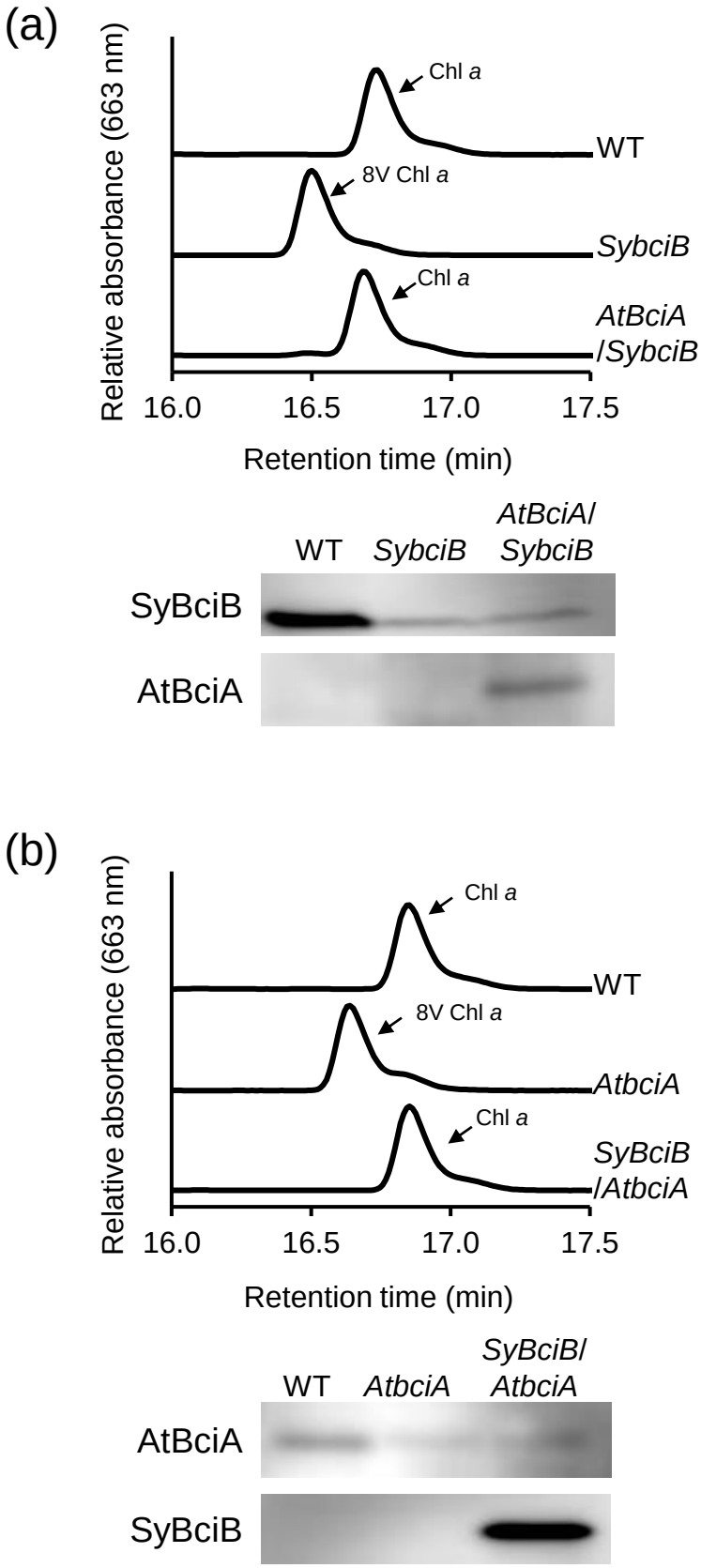
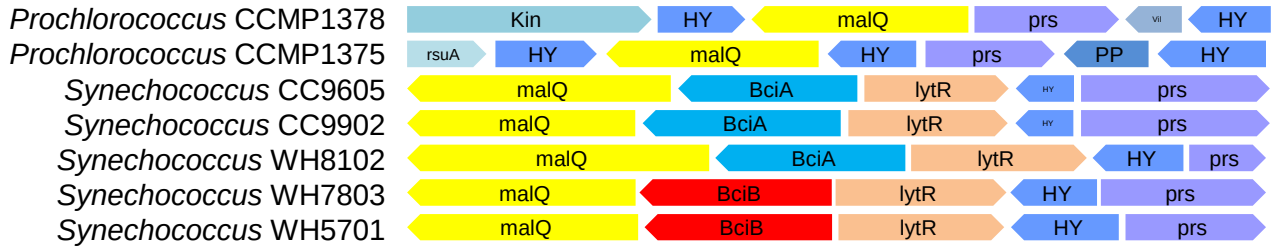

(a)
Relative absorbance (663 nm)
Chl a
WT
8V Chl a
SybciB
Chl a
AtBciA
/SybciB
16.0
16.5
17.0
17.5
Retention time (min)
WT
SybciB
AtBciA/
SybciB
SyBciB
AtBciA
(b)
Relative absorbance (663 nm)
Chl a
WT
8V Chl a
AtbciA
Chl a
SyBciB
/AtbciA
16.0
16.5
17.0
17.5
Retention time (min)
WT
AtbciA
SyBciB/
AtbciA
AtBciA
SyBciB

Fig. 6

8-Vinyl reductase



SOD