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Title page

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Title: Evolution of a divinyl chlorophyll-based photosystem in *Prochlorococcus*

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ABSTRACT

Acquisition of new photosynthetic pigments has been a crucial process for the evolution of photosynthesis and photosynthetic organisms. In this process, pigment-binding proteins must evolve to fit new pigments. *Prochlorococcus* is a unique photosynthetic organism that uses divinyl chlorophyll (DVChl) instead of monovinyl chlorophyll (MVChl). However, cyanobacterial mutants that accumulate DVChl immediately die even under medium-light conditions, suggesting that chlorophyll (Chl)-binding proteins had to evolve to fit to DVChl concurrently with *Prochlorococcus* evolution. To elucidate the co-evolutionary process of Chl and Chl-binding proteins during the establishment of DVChl-based photosystems, we first compared the amino acid sequences of Chl-binding proteins in *Prochlorococcus* with those in other photosynthetic organisms. Two amino acid residues of the D1 protein, V205 and G282, are conserved in MVChl-based photosystems, but in *Prochlorococcus*, they are substituted with M205 and C282, respectively. According to the solved photosystem II structure, these amino acids are not involved in Chl binding. In order to mimic *Prochlorococcus*, V205 was mutated to M205 in the D1 protein from *Synechocystis* sp. PCC6803 and *Synechocystis dvr* mutant was transformed with this construct. Although these transgenic cells could not grow under high-light conditions, they acquired light tolerance and grew under medium-light conditions, whereas untransformed *dvr* mutants could not survive. Substitution of G282 for C282 contributed additional light tolerance, suggesting that the amino acid substitutions in the D1 protein played an essential role in the development of DVChl-based photosystems. Here, we discuss the co-evolution of a photosynthetic pigment and its binding protein.

Introduction

Photosynthesis is one of the most important biological processes, contributing not only to biological activity but also to maintaining the global environment (1). The apparatuses and properties of photosynthesis have been dynamically changed during evolution (2). The first photosynthetic organisms are thought to be anaerobic photosynthetic bacteria, which did not produce oxygen in the process of photosynthesis (3). Cyanobacteria were the first oxygenic phototrophs whose photosystems evolved from those of preexisting photosynthetic bacteria (4, 5). The conversion of bacteriochlorophyll to Chl *a* was crucial, even in this evolutionary process, because it permitted the oxidation of H₂O (3). Besides this event, the acquisition of new photosynthetic pigments has greatly contributed to the diversification of photosynthetic organisms (6). All photosynthetic pigments except chlorosomes exist as pigment-protein complexes; therefore, acquisition of the binding protein for the new pigment has been an indispensable process in the establishment of new photosynthetic pigment systems. Because photosynthetic organisms cannot acquire the pigment-binding protein in advance, a new pigment would first need to be incorporated into preexisting proteins after which the binding proteins would evolve to better fit the new pigment (7). To understand the evolution of pigment systems, phylogenetic analyses of pigment-synthesis enzymes (8) and pigment-binding proteins (9) have been extensively performed. However, the co-evolutionary process of a pigment and its binding protein cannot be elucidated by this method due to the lack of information about the intermediate states of the pigment-protein complexes, which appeared and then disappeared during evolution. *In vivo* experiments that mimic the evolution of the pigment-protein complexes are a potentially powerful tool that could be used to overcome this problem (7).

Prochlorococcus belongs to a marine picophytoplankton clade (10, 11) and is a major participant of the global carbon cycle (12), in part due to its unique photosynthetic system, which uses DVChl instead of MVChl (13). DVChl allows *Prochlorococcus* to photosynthesize and grow under the deep-sea water column where blue light predominates because DVChl harvests blue light more efficiently than MVChl. Thus, acquisition of DVChl was an important

evolutionary event for *Prochlorococcus* (14). Recently, we identified the 3,8-divinyl chlorophyllide 8-vinyl reductase (DVR) genes, which convert a vinyl group on the C-8 position to an ethyl group, in *Arabidopsis* (15) and cyanobacteria (16). Genome analysis showed that marine *Synechococcus*, a closely related species, contains a DVR gene while *Prochlorococcus* does not, indicating that the progenitor of the *Prochlorococcus* genus lost the DVR gene and acquired DVChl. However, cyanobacterial and *Arabidopsis dvr* mutants, which accumulate DVChl instead of MVChl, immediately die under high-light conditions due to severe photodamage and can survive only under low-light conditions (16). In contrast, *Prochlorococcus* adapted to high-light conditions despite the presence of DVChl (17). One possible mechanism for this adaptation is the evolution of Chl-binding proteins to fit to DVChl within the *Prochlorococcus* lineage.

To elucidate the evolution of DVChl-based photosystems, we first compared the amino acid sequences of Chl-binding proteins of MVChl-based and of DVChl-based photosystems. We found two amino acid residues that are conserved only in *Prochlorococcus* D1 proteins and could not find any *Prochlorococcus*-specific amino acid residues in other Chl-binding proteins. When these two amino acid residues of *Synechocystis* D1 proteins were substituted with the residues found in *Prochlorococcus* and introduced into a *Synechocystis dvr* mutant, the transgenic *Synechocystis* acquired light tolerance. In this paper, we discuss the co-evolutionary process of a photosynthetic pigment and its binding protein.

Results

Photodamage of DVChl-containing organisms. MVChl and DVChl have an ethyl group and a vinyl group at position C8, respectively (Fig. 1A). When DVR is mutated or lost during evolution, the final product of Chl biosynthesis is DVChl. The *Arabidopsis* and *Synechocystis dvr* mutants synthesize DVChl and construct DVChl-based photosystems. These mutants grow photosynthetically under low-light conditions. When low-light-grown *Arabidopsis* or cyanobacteria were transferred to high-light conditions, wild type survived, but both *dvr* mutants lost Chl and died within one day (Fig. 1B). One possible mechanism for the rapid degradation of Chl is the induction of Chl degradation enzymes in *dvr* mutants. The other is the severe photodamage under high-light conditions in *dvr* mutants, although light harvesting systems differ between the two organisms (14).

Comparison of amino acid sequences of core complexes. DVChl is structurally different from MVChl, as shown in Fig. 1, and this structural variation may prevent DVChl from fitting into MVChl binding sites, resulting in severe photodamage in *dvr* mutants. The question then arises as to which Chl-protein complexes caused photodamage in the *dvr* mutants. *Arabidopsis* and *Synechocystis* contain different peripheral antenna systems, but the core antenna and reaction center complex are well conserved in both organisms, suggesting that the core antenna and/or reaction center complexes cause the common photodamage observed in both of the *dvr* mutants. Because *Prochlorococcus* uses DVChl instead of MVChl, we sought to identify changes in the Chl-binding proteins specific to *Prochlorococcus* by aligning the amino acid sequences of the reaction center complexes of photosystem II (D1, D2), the core antenna complexes of photosystem II (CP43, CP47) and the P700-Chl *a*-protein complex (CP1). We could not find any *Prochlorococcus* specific amino acid residues in D2, CP43, CP47 and CP1, suggesting that these core complexes can accept DVChl without modifications. In contrast, D1 proteins of all *Prochlorococcus* strains (18) contain two conserved amino acid residues (M205 and C282) that are generally substituted with V205 and G282 in other organisms including marine *Synechococcus* strains (19) (Fig. S1 and Discussion).

Substitution of D1 protein. If the evolution of the D1 protein is a crucial process for acquiring DVChl-based photosystems, the substitution of *Synechocystis* D1 by *Prochlorococcus* D1 should increase the light tolerance of *Synechocystis dvr* mutants. To test this hypothesis, we introduced the *Prochlorococcus psbA* gene into a *Synechocystis dvr* mutant. Three *psbA* genes in the *Synechocystis* genome were disrupted, and modified *psbA* was introduced. These transgenic lines were used to investigate the function of the introduced *Prochlorococcus psbA* gene. In order to compare the transgenic lines with wild type and the *dvr* mutants, the *psbA1* and *psbA3* genes in these two lines were disrupted. The MVChl-accumulating *Synechocystis* (MV mutant), DVChl-accumulating *dvr* mutant (DV mutant) and DV mutant harboring the *Prochlorococcus* D1 (DVPro mutant) all grew well under heterotrophic conditions. However, the DVPro mutant could not grow phototrophically (Fig. 2A), indicating that the *Prochlorococcus* D1 protein does not fit into the *Synechocystis* photosystem II. Next, instead of switching the whole D1 protein, only V205 or G282 or both residues of the *Synechocystis* D1 protein (PsbA2) were mutated to Met and Cys, respectively, and this protein was introduced into the *Synechocystis* DV mutant (Fig. S2). Substitution of one or two amino acid residues might not cause drastic structural changes to the D1 protein. Transgenic lines carrying the modified D1 protein grew phototrophically (Fig. 2B). A typical phenomenon of photodamage in the *dvr* mutant is the rapid degradation of Chl under high-light conditions, as shown in Fig. 1B. To examine whether the amino acid substitutions protect against photodamage, low-light-grown cells were exposed to high light ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 6 h, and the Chl contents were determined. Chl did not decrease in the MV mutant after high-light treatment, but approximately 60% of the Chl degraded in the DV mutant. Interestingly, the V205M/G282C and G282C mutants retained 80% of the initial level of Chl, whereas 40% of the Chl degraded in the V205 M mutant during high-light treatment.

Growth rates and photosynthetic capacity of transformed cells. In many cases, substitution of amino acid residues in the D1 protein impairs photoautotrophic growth due to the loss of photosynthetic capacity (20) because many amino acid residues participate in water oxidation, charge separation and other important processes of photosystem II. To examine the impact of the amino acid substitution of the D1 protein, the growth of the transformants were assessed by optical density at 750 nm, cell numbers and Chl levels under low ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) or medium ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$) light conditions (Fig. 3A and Fig. S3). All of the cell lines grew well under low-light conditions. Under medium-light conditions, the MV, V205M/G282C and V205M mutants could grow, but the DV and G282C mutants could not proliferate (Fig. 3A). Growth impairment of the G282C mutant is inconsistent with the observation that Chl was more stable in the G282C mutant than in the V205M mutant upon strong illumination (Fig. 2B). It is possible that growth impairment under medium light (Fig. 3A) might be caused by a distinct mechanism from that which influences the stability of Chl under strong illumination (Fig. 2B). Although the optical density of the MV mutant was slightly higher than that of other lines after 5 days of medium light, the doubling times of the MV, V205M/G282C and V205M mutants calculated from Fig. 3A were 1.18, 1.27 and 1.29 days. Optical densities and cell numbers exhibited almost the same profiles (see Fig. 3A and Fig. S3A). Analysis of the growth of the transformants under low- and medium-light conditions indicates that the substitution of the two amino acid residues played an essential role in the development of a DVChl-based photosystem. In order to examine the effect of the amino acid substitution on the growth of *Synechocystis* which uses MVChl-based photosystems, V205 and G208 were substituted for M205 and C282, respectively, in MV mutant (MV-V205M/G282C mutant). MV and MV-V205M/G282C mutants exhibited the same growth profile (Fig. S4), indicating that substitutions of these two amino acid residues have no impact on

MVChl-based photosystems.

Next, we measured the increase in biomass of the MV, DV and V205M/G282C mutants during incubation under various light intensities to examine to what extent these amino acid substitution contributes to light tolerance (Fig. 3B). The DV mutant was unable to grow under $100 \mu\text{mol m}^{-2} \text{s}^{-1}$. The V205M/G282C mutant grew under $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ but it died under $500 \mu\text{mol m}^{-2} \text{s}^{-1}$. In contrast, the MV mutant survived even under $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Although these amino acid substitutions significantly enhanced the light tolerance of DVChl-based photosystems, these photosystems are still less tolerant to high-light intensities than MVChl-based photosystems. In order to examine whether amino acid substitutions contribute to further light tolerance of the MVChl-based photosystems, MV-V205M/G282C mutant was grown under high-light conditions. Optical density of MV-V205M/G282C mutant decreased under $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ compared to $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ as MV mutant (Fig. 3B), indicating that these amino acid substitutions have no effect to light tolerance of MVChl based photosystems.

The O_2 evolution per Chl of low-light-grown cells was determined under saturating light intensity (Fig. 4A). O_2 evolution rate was low in the V205M mutant but high in the G282C mutant. After 5 days of medium light, the rates of O_2 evolution of the MV, V205M/G282C and V205M mutants were 290, 168 and 115 $\mu\text{mol mg Chl}^{-1} \text{h}^{-1}$, respectively, but those of the DV and G282C mutants were under detectable levels (Fig. 4B). These results of O_2 evolution are consistent with the optical density after incubation for 5 days (Fig. 3A).

Next, we examined whether amino acid substitutions reduce the photoinhibition in the transformants. Cells were exposed to various light intensities in the presence of lincomycin which inhibits the repair process of photosystem II (Fig. 4C). The rate of O_2 evolution decreased by about 60 and 30% in DV and V205M/G282C mutants, respectively, after 3 h of light treatment ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), indicating that the amino acid substitutions decrease the extent of photoinhibition. The amino acid substitutions did not have much of an impact at higher light intensities of 250 and $500 \mu\text{mol m}^{-2} \text{s}^{-1}$. These results suggest that higher tolerance of the amino acid substituted lines against medium light can be at least partly explained by decreased rates of photoinhibition.

Taken together, all strains grow at low light and evolve oxygen, but at medium light neither the DV nor the G282C mutants grow nor evolve oxygen (Fig. 3 and Fig. 4). Both V205M and G282C substitutions in the D1 protein of the DV mutant suppressed Chl degradation under high-light conditions (Fig. 2B).

Discussion

Evolution of a DVChl-based photosystem. Although molecular phylogenetic analysis is a powerful tool for understanding the evolution of photosynthesis and photosynthetic organisms, the biochemical processes of the evolution cannot be uncovered by this method (7). However, we can deduce the evolutionary intermediates of photosynthetic machineries based on the comparison of genome sequences. Additionally, we can produce the organism that will use these photosynthetic machineries (21), and examine the physiological responses of this organism. Here, we employed this method for understanding the evolution of the DVChl-based photosystem of *Prochlorococcus*.

Phylogenetic analysis of 16S rRNA has shown that *Prochlorococcus* is phylogenetically closely related to marine *Synechococcus* (10). Genomic analyses of *Synechococcus* and *Prochlorococcus* revealed that the progenitor of the genus *Prochlorococcus* lost the DVR gene and acquired DVChl (15). This progenitor had an advantage in that it could efficiently absorb blue light, which is enriched in deep-water layers. However, this progenitor also had a disadvantage because it could survive only under low-light conditions, as suggested by the *dvr* mutant. We hypothesize that the first step in the acquisition of high-light tolerance was the substitution of the amino acid residues in the D1 protein.

Support for this idea is based on the finding that M205 and C282 of D1 are unique amino acid residues that are conserved only in *Prochlorococcus* D1 proteins. We could not find *Prochlorococcus* specific sequences/residues in other Chl binding proteins. This strongly suggests that these two amino acids were mutated to the *Prochlorococcus* type (M205 and C282) in a common progenitor of the genus *Prochlorococcus*. Then, this progenitor acquired significant tolerance to high-light conditions, despite the accumulation of DVChl, which is normally toxic to cells under high-light conditions. Another hypothesis is that the substitutions of these amino acids were followed by the conversion of MVChl to DVChl in the ancestor of *Prochlorococcus*. This hypothesis is consistent with the finding that the strain with the altered D1 functioned well in MVChl-based photosystems (Fig. 3B and Fig. S4). Either way, these changes to the D1 protein appear to be a crucial step in the establishment of DVChl-based photosystems.

Between the two amino acid residues, the substitution of V205 to M205 might be more important, as the V205M mutant could grow under medium-light conditions and evolved oxygen. The substitution of G282 for C282 results in less Chl degradation, which indicates that the substitution of G282 for C282 contributed to additional light tolerance (Fig. 2B). Other Chl-binding proteins might tolerate DVChl because photoinhibition occurs mainly in the reaction center of photosystem II and because D1 proteins are targets of photodamage. In addition to these two residues, various other amino acid substitutions in the D1 protein occurred independently in each of the *Prochlorococcus* lineages. Some of these substitutions might contribute to a better fit with DVChl. The *Prochlorococcus* genus contains both species adapted to high-light conditions and species adapted to low light (17, 22, 23). Low-light-adapted *Prochlorococcus* lives under low-light intensities to which the *dvr* mutant can tolerate, suggesting that this *Prochlorococcus* can survive in nature without amino acid substitutions. However, it is known that photodamage is enhanced by other physiological stresses (24). Furthermore, low-light-adapted *Prochlorococcus* has a large antenna size. Acquisition of stress tolerance might be beneficial even for low-light-adapted *Prochlorococcus*. The hypothesis cannot be excluded that high-light adapted *Prochlorococcus* acquired more light tolerance through additional changes in the amino acid sequence of the D1 protein. *Prochlorococcus* ecotypes might have adapted their D1 protein to specific cellular and environmental conditions to protect against photodamage. In addition to the evolution of D1 protein, decrease in photosynthetic antenna size might also be an important process for the acquisition of light tolerance during the evolution of high-light adapted *Prochlorococcus* (25).

Most of the MVChl-based photosynthetic organisms contain the conserved V205 and G282 residues. However, *Acaryochloris marina*, which uses Chl *d* as a photosynthetic pigment (26), retains C282 instead of G282, although V205 is conserved (Fig. S1). One possible reason for this amino acid substitution in *Acaryochloris* is to protect against photodamage by accommodating Chl *d*, which is structurally different than Chl *a* (27, 28).

Mechanism of two amino acid residues for protection against photodamage. Photodamage in DV mutants is not caused by defects in energy dissipation in the antenna system but rather by the reaction center of photosystem II (29) because the substitution of amino acid residues in the D1 protein led to a partial recovery from photodamage of the DV mutant. This is consistent with the report that D1 protein is more rapidly degraded by high-light treatment in *dvr* mutants as compared with wild type. Antenna systems potentially have the flexibility to accept structurally different pigments; for example, *Synechocystis* can use Chl *b* as a photosynthetic pigment (21, 30), and core antenna complexes of both photosystems can bind Chl *b* (31), although these proteins form Chl *a*-protein complexes in nature. In contrast, the reaction center complex might have many structural restrictions because the complex is responsible for many functions, such as charge separation, energy and electron transfer and water oxidation. The atomic structure of

photosystem II of cyanobacteria shows that V205 is near the reaction center (32), which enables V205 to interact with a “special pair” (33) (Fig. S5). It is predicted that V205 is not involved in binding the special-pair Chl and can not interact with pheophytin (32). V205 also does not directly interact with a vinyl group on pyrrole ring B but with other moiety of special-pair Chl. When V205 is replaced by Met, interactions of the residue with special-pair Chl and pheophytin becomes stronger due to a larger side chain of Met. If the replacement of this amino acid residue alters the potential of the special pair and/or pheophytin of photosystem II due to the strong interaction, the photodamage would be reduced (34). Although photoinhibition rate was reduced in the V205M/G282C mutant, it cannot be excluded the possibility that other processes such as the repair cycle and other unknown process contribute to light tolerance of the V205M/G282C mutant. Although the mechanisms underlying the induction of and protection from photodamage have not yet been elucidated, the study of the transformed cells from this report will contribute to our understanding of these mechanisms of reaction-center photoinhibition in cyanobacteria. It should be noted that these two amino acid substitutions contribute to survive under medium-light condition but not under high-light condition. In order for *Prochlorococcus* to become high-light adapted, further processes would be required, for example, evolution of high-light inducible gene family (35, 36).

Distribution of *psbA* genes in cyanophages. Photosynthesis genes exist not only in photosynthetic organisms but also in cyanophages (37-39). Some podoviruses and myoviruses infecting *Prochlorococcus* and *Synechococcus* contain *psbA* genes. It was reported that these cyanophages acquired *psbA* genes from *Prochlorococcus* and *Synechococcus* during evolution (40). With the two exceptions of P-SSM1 and S-ShM1 (40), *Synechococcus* myoviruses and *Synechococcus* podoviruses isolated from *Synechococcus* encode MVChl-type D1 proteins, whereas *Prochlorococcus* myoviruses contain DVChl-type D1 proteins. This indicates that cyanophages infecting *Prochlorococcus* and *Synechococcus* contain DVChl- and MVChl-type D1 proteins, respectively. This finding is consistent with the idea that cyanophage *psbA* genes contribute to host photosynthesis.

However, this is not a strict rule with respect to cyanophage genes, as observed in *Prochlorococcus* podovirus, which contains the *psbA* gene and infects *Prochlorococcus* (41). Although the *psbA* gene of *Prochlorococcus* podovirus is phylogenetically close to the *Prochlorococcus* *psbA* genes, podovirus D1 proteins contain the MVChl-type residues V205 and G282. It was also reported that some phages can infect both *Prochlorococcus* and *Synechococcus* (41). These observations contradict the idea that the phage *psbA* gene is expressed during infection and contributes to photosynthesis within the host cells (42, 43) because MVChl-type D1 proteins cause photodamage in the DVChl-containing photosystem. Furthermore, *Prochlorococcus* podoviruses predominantly infect high-light-adapted *Prochlorococcus*. One possibility is that the *psbA* gene does not contribute to photosynthetic capacity but has another unknown function. Another possibility is that D1 protein of these phages evolved to fit to *Prochlorococcus* and/or *Synechococcus* by altering amino acid sequence other than the two conserved amino acids (205 and 282) or by another protein from phage, which enable D1 to function in cyanobacteria. The true function of the phage *psbA* gene still needs to be examined.

Phylogenetic analysis of the *psbA* genes of cyanobacteria and cyanophages indicated that *Prochlorococcus* podovirus acquired the *psbA* gene from a common ancestor of the *Prochlorococcus* genus (Fig. S6) (40). There are two hypotheses concerning the Chl type of this ancestral *Prochlorococcus* from which podovirus acquired the *psbA* gene. The first hypothesis is that the ancestor had already acquired DVChl, and the second is that this ancestor still used MVChl. As shown in this report, MVChl-based photosynthetic organisms are severely photodamaged if they

have DVChl-type D1 proteins. If the *Prochlorococcus* progenitor changed the D1 protein to the DVChl type immediately after acquiring DVChl to protect against photodamage, *Prochlorococcus* podovirus should have acquired the *psbA* gene from ancestral *Prochlorococcus* that still used MVChl. The study of *psbA* genes in cyanophage will contribute to our understanding of the evolution of *Prochlorococcus*.

MATERIALS AND METHODS

Materials and growth conditions. In this report, low, medium and high light indicate 30, 100-250 and 500-1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. *Arabidopsis thaliana* (Columbia ecotype) was grown at 23°C under continuous light in a chamber equipped with white fluorescent lamps at a light intensity of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For high-light treatment, 3-week old *Arabidopsis* plants were transferred to high light (1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 1 day.

Synechocystis sp. PCC6803 cells were grown at 30°C in liquid BG11 medium with ambient CO_2 under continuous illumination (30 $\mu\text{mol m}^{-2} \text{s}^{-1}$) rotating at 120 rpm, and the logarithmically growing cells were used for further experiments. For the experiments of light-induced Chl degradation, the OD750 of the liquid cell cultures was set to 1.0 (2 mL cell culture in a 24-well plate) for Fig. 1B and 0.5 for Fig. 2B, and the cells were exposed to 500 or 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 30°C. For the growth experiments (Figs. 3 and 4A), the OD750 of the liquid cell cultures was set to 0.05 (100 mL cell culture in a 200 mL flask) and the cells were grown under various light intensities at 30°C.

Constructs and transformations. All *Synechocystis* transformants analyzed in this study were prepared from *Synechocystis* engineered to express a 6×histidine-tag at the C-terminus of CP47 selected by coexpressing the kanamycin resistance gene. Furthermore, all transformants were prepared by the disruption of *psbA1* and *psbA3* by replacing part of the coding region by a cassette encoding an erythromycin and a gentamycin resistance gene via homologous recombination. For generating the V205 M and G282C site-directed mutants, GAT (codon 613-615) and GGC (codon 844-846) were changed to ATG and TGC, respectively, and the transformants harboring mutated *psbA2* were selected by coexpressing the streptomycin resistance gene (44, 45). Segregation of the mutant genomes was confirmed by PCR (Fig. S2). Furthermore, the sequence analysis of the cDNA that were reverse-transcribed from *psbA* mRNA confirmed that only modified *psbA* gene was expressed in these transformants (Fig. S2). The *slr1923* locus encoding 3,8-divinyl chlorophyllide 8-vinyl reductase was disrupted by the chloramphenicol resistant cassette as reported previously (16).

Flow Cytometry Analysis. Cell numbers of transformants were monitored by flow cytometry using a FACS Canto flow cytometer with a 488-nm laser (BD Biosciences). Culturing cells were directly loaded with the acquisition performance at a low rate (10 $\mu\text{L min}^{-1}$). Bleached cells generated under medium-light conditions were taken into account to estimate the number of cells.

Chl Measurement. One mL of culture was centrifuged at 22,000g for 5 min to precipitate cells. Cells were resuspended in methanol to extract Chl. After centrifugation at 22,000g for 5 min, the absorbance at 665 nm of the supernatant was measured and Chl content was calculated following the equations by Porra (46).

Measurement of Oxygen Evolution. Photosynthetic oxygen-evolving activity of intact cells was measured using a Clark-type oxygen electrode (Hansatech Instruments). Cell suspension (1 mL) was put into the measurement chamber

of the oxygen electrode and oxygen evolution rates were measured under saturating light ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 2 min at 30°C in the presence of 5 mM NaHCO_3 . For the experiment of Fig. 4A, cells exponentially growing under low-light conditions ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) were suspended in BG11 medium to $10 \mu\text{g Chl mL}^{-1}$ and oxygen evolution rates were measured. To evaluate the photosynthetic activity of the cells growing under medium-light conditions, cells were cultured for 5 days at $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ as in Fig. 3A and oxygen-evolving activity was measured (Fig. 4B). For the photoinhibition experiments (Fig. 4C), cells growing under low light were exposed to various light intensities and the oxygen-evolving activity was measured.

Photoinhibition treatment. Low-light grown *Synechocystis* cells were suspended in 2 mL BG11 medium ($\text{OD}_{750}=1.0$) and transferred into a 24-well plate. In order to measure the photoinhibition under various light intensities, cells were exposed to 30, 100, 250 or $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 3 h in the presence of $300 \mu\text{g mL}^{-1}$ lincomycin which inhibits repair processes in the photosystem II reaction center.

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Figure Legends

Fig. 1. DVChl-accumulating mutants in *Arabidopsis* and cyanobacterium.

(A) Structure of DVChl and MVChl. The positions of the 8-vinyl group and 8-ethyl group are indicated by a circle on each chemical structure. (B) Photodamage of *Arabidopsis* and *Synechocystis dvr* mutants accumulating DVChl. *Arabidopsis* mutants were grown for 3 weeks under standard light conditions ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$) and exposed to strong light ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 24 h. *Synechocystis* grown under low-light conditions ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) was suspended in culture medium ($\text{OD}_{750}=1.0$) and exposed to strong light ($500 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 24 h.

Fig. 2. Effect of the substitution of D1 protein on photodamage.

(A) Phototrophic and heterotrophic growth of transformants. MVChl-accumulating cell (MV mutant), DVChl-accumulating cell (DV mutant) and DVChl-accumulating cell whose D1 protein is substituted with the *Prochlorococcus* D1 protein (DVPro mutant) were cultured on agar. *psbA1* and *psbA3* of all *Synechocystis* cells used in this study were disrupted. Cells were grown phototrophically under continuous illumination ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) or heterotrophically with 5 mM glucose under dim light ($5 \mu\text{mol m}^{-2} \text{s}^{-1}$) on agar plates for 1 week. (B) Chl degradation under high-light conditions. Cells grown photoautotrophically under low-light conditions ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) were suspended in the BG11 medium ($\text{OD}_{750}=0.5$) and incubated at 30°C for 6 h under high light ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$). Before (white bar) and after (black bar) high-light treatment, Chl was extracted from the cells and measured by the absorbance at 663 nm. The change in Chl content is expressed as the ratio of the absorbance of Chl extracted after high-light treatment to the absorbance of Chl extracted before treatment. Error bars represent the standard deviations based on the mean values of three samples. *Values differ significantly from the DV mutant after high-light treatments ($P<0.01$ by *t* test). **Values differ significantly from the G282C mutant after high-light treatments ($P<0.05$ by *t* test). MV, MV mutant; DV, DV mutant; V205M/G282C, V205M/G282C mutant; V205M, V205 M mutant; G282C, G282C mutant.

Fig. 3. Cell growth under different light conditions.

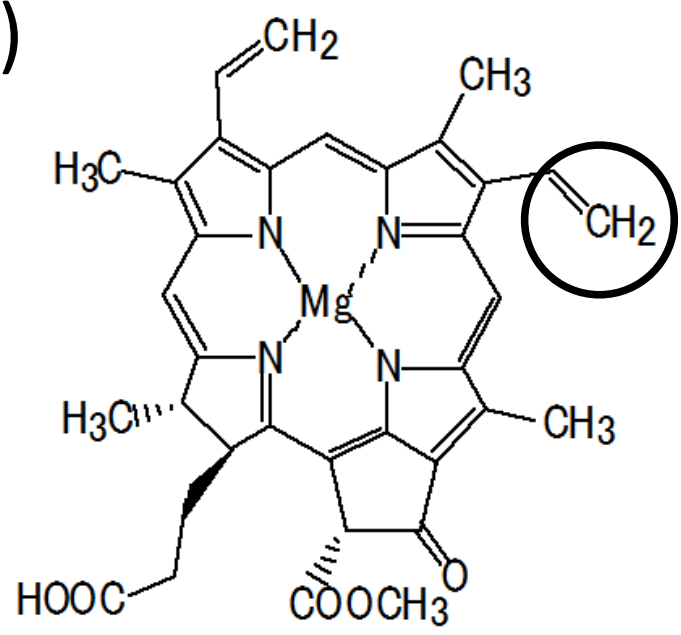
(A) Cell growth under low or medium light conditions. Cells growing under low light ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) were diluted with culture medium to $\text{OD}_{750}=0.05$ and grown under low ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) or medium ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$) light conditions, rotating at 30°C . Cell biomass was monitored by measuring optical density at 750 nm. (B) Effect of light intensity on cell growth. Low-light-grown diluted cells ($\text{OD}_{750}=0.05$) were incubated under 30, 100, 200, 500, 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After incubation for 3 days, OD_{750} , which is used as a proxy for biomass, was measured. Error bars represent the standard deviations based on the mean values of three samples. MV, MV mutant; DV, DV mutant; V205M/G282C, V205M/G282C mutant; V205M, V205 M mutant; G282C, G282C mutant; MV-V205M/G282C, MV-V205M/G282C mutant.

Fig. 4. Photosynthetic oxygen-evolving activity of transformants.

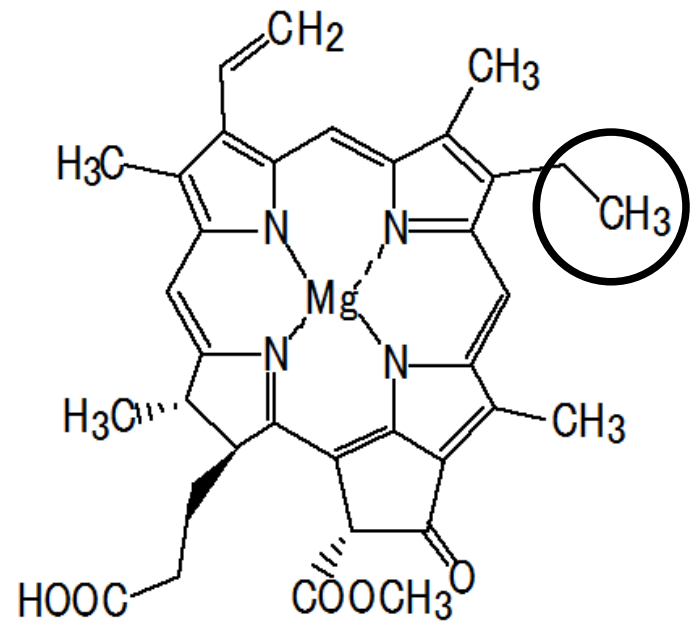
(A) Oxygen evolution rate of low-light-grown cells. Cells growing under low-light conditions ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) were suspended in BG11 medium to $10 \mu\text{g Chl mL}^{-1}$ and oxygen evolution was measured. (B) Oxygen evolution rate of medium-light-grown cells. Cells were grown under medium-light ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 5 days as in Fig. 3A and oxygen evolution rate was measured. N. D.; not detected. (C) Effects of light intensity on photoinhibition. Cells growing under low-light conditions ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) were suspended in culture medium at a concentration of

OD₇₅₀=1.0 and incubated under 30, 100, 250, 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 3 h at 30°C in the presence of lincomycin (300 $\mu\text{g mL}^{-1}$). Before and after light treatment, oxygen evolution was measured. Error bars represent the standard deviations based on the mean values of three samples. Photosynthetic oxygen-evolving activity of intact cells was measured using a Clark-type oxygen electrode at 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the presence of 5 mM NaHCO₃ at 30°C. MV, MV mutant; DV, DV mutant; V205M/G282C, V205M/G282C mutant.

(A)

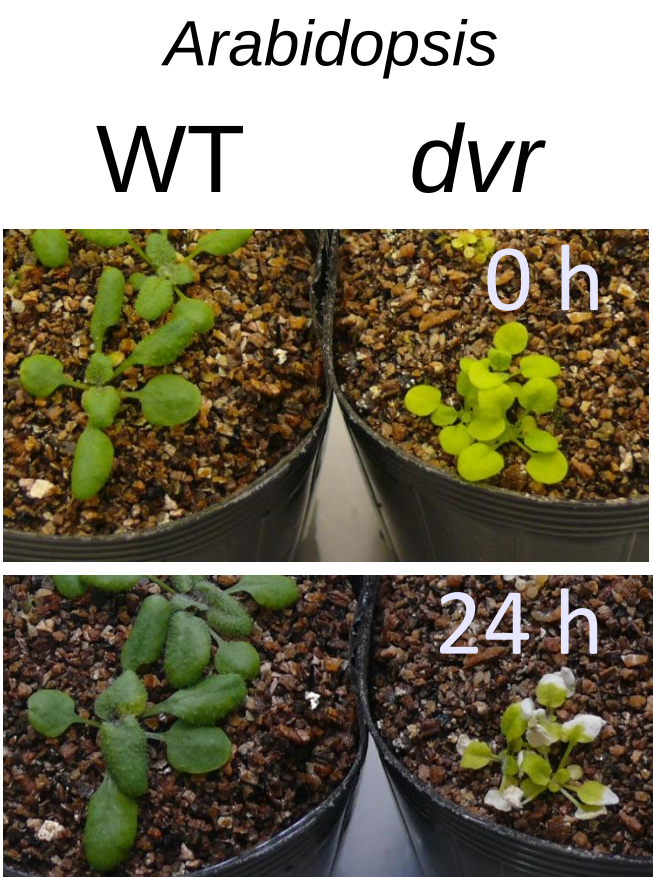


3,8-Divinyl chlorophyllide a

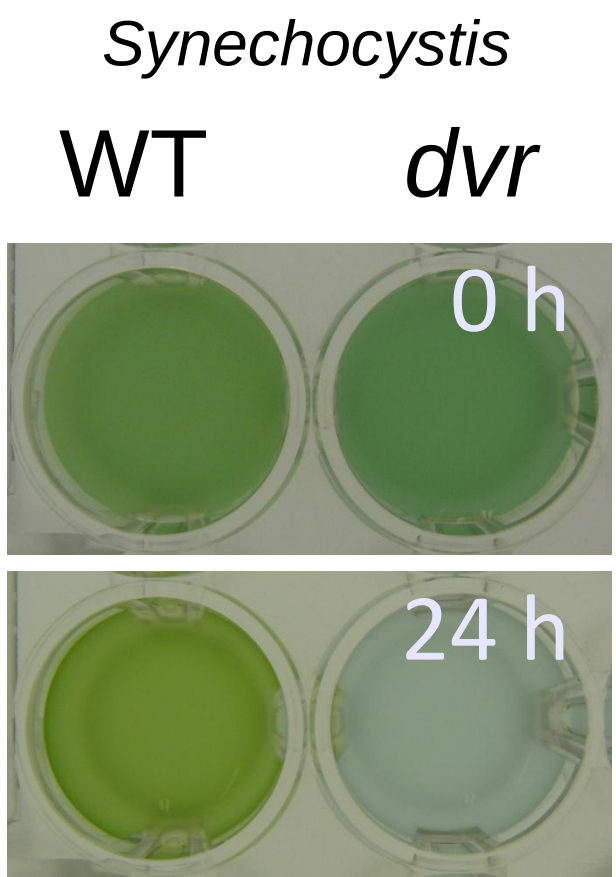


Monovinyl chlorophyllide a

(B)



1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$



500 $\mu\text{mol m}^{-2} \text{s}^{-1}$

Fig. 1

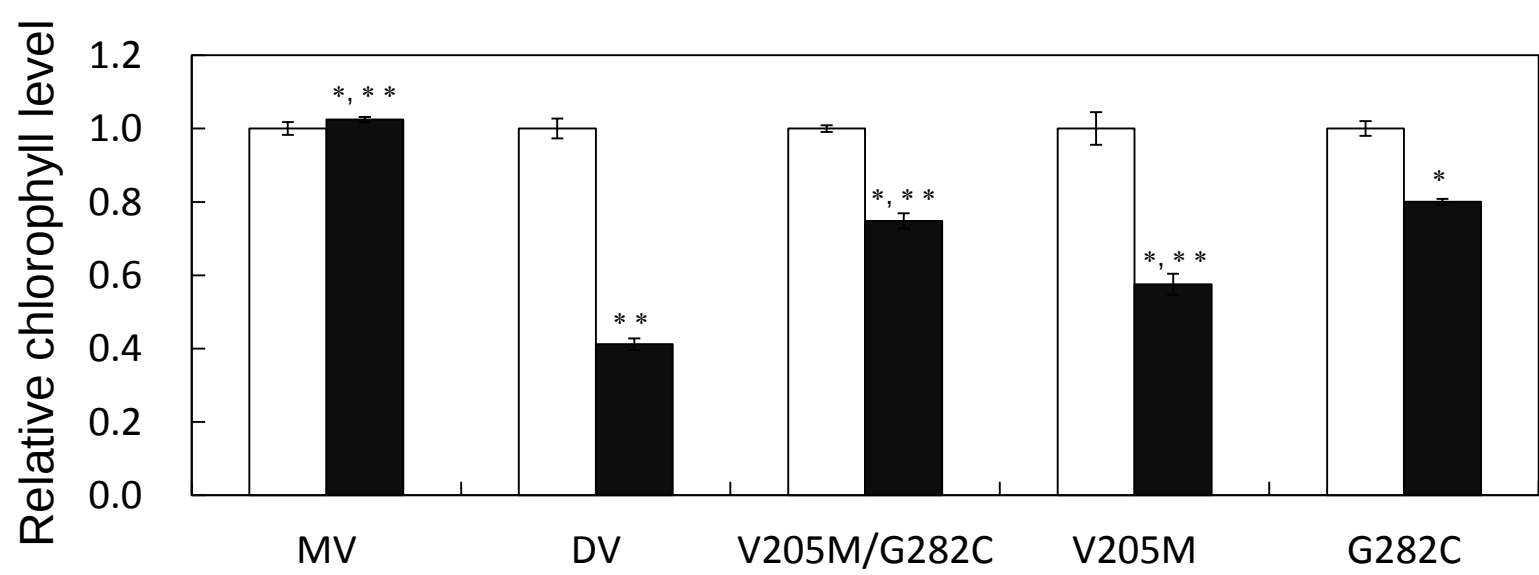
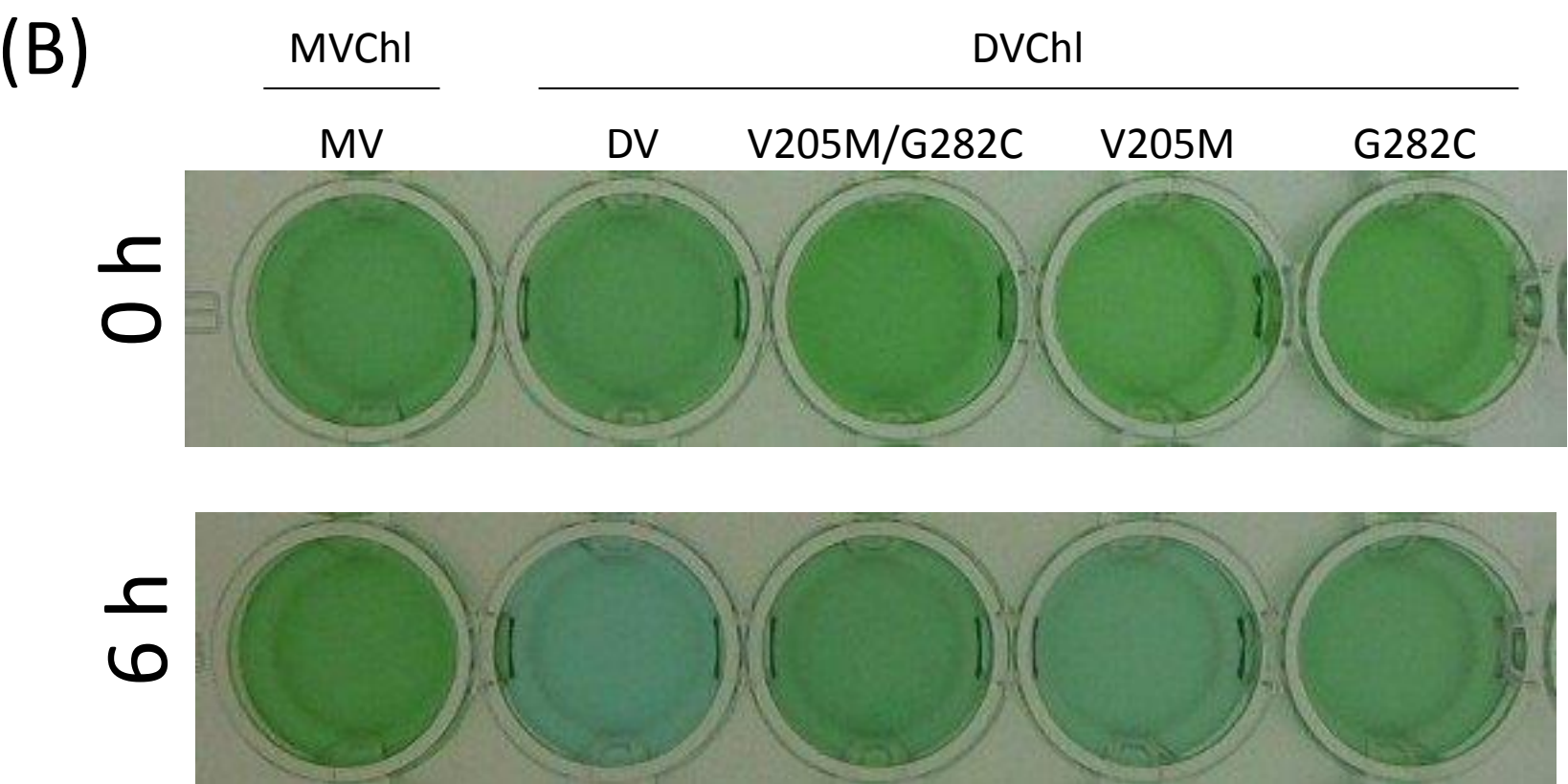
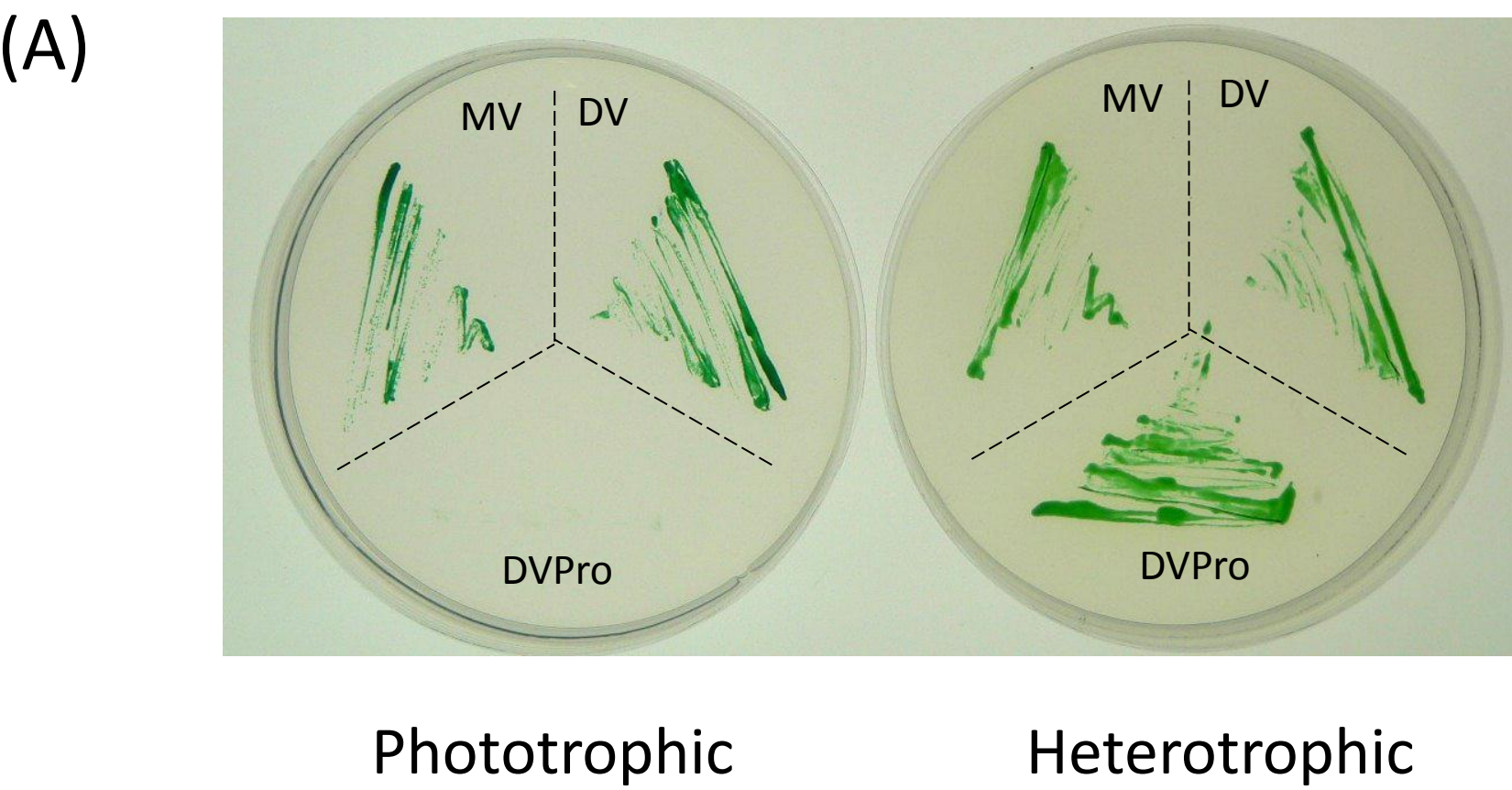


Fig. 2

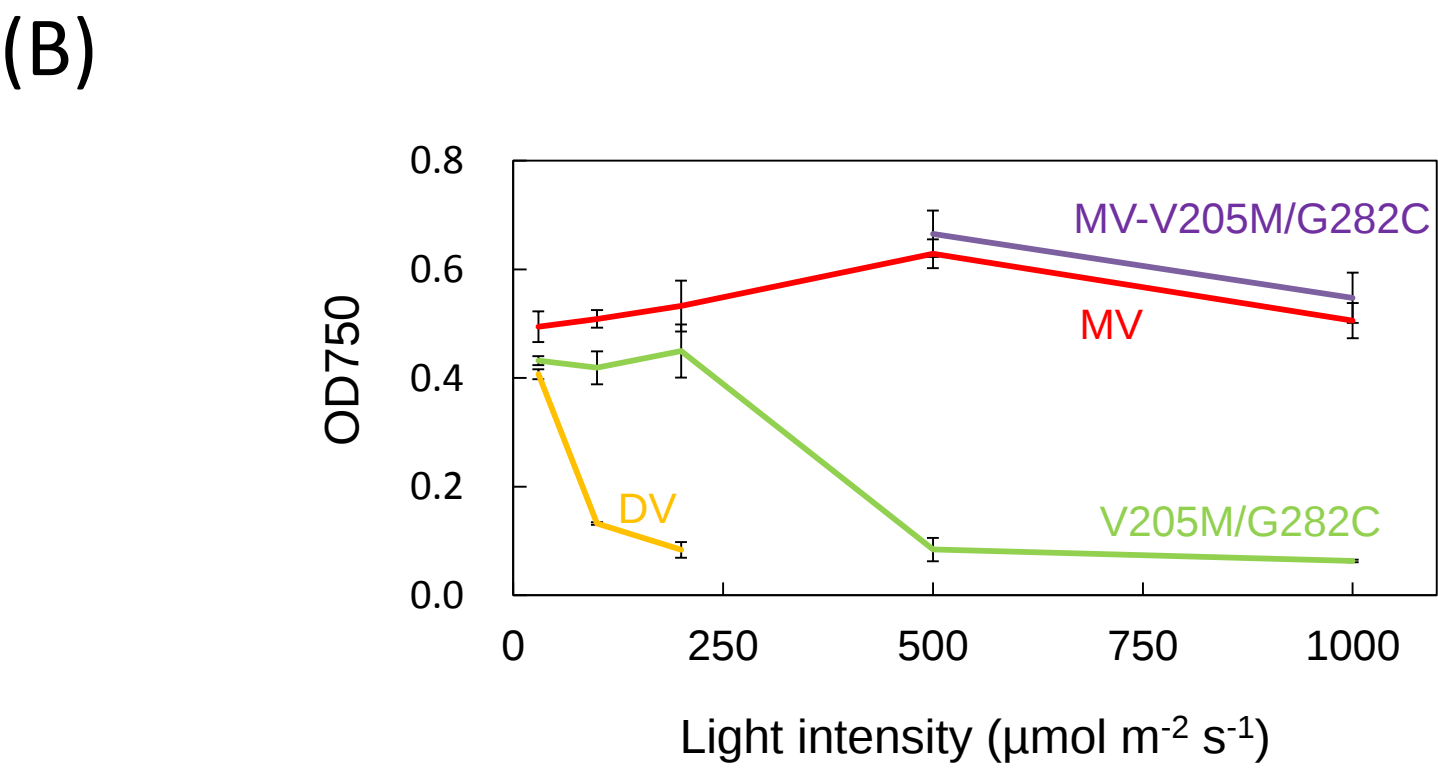
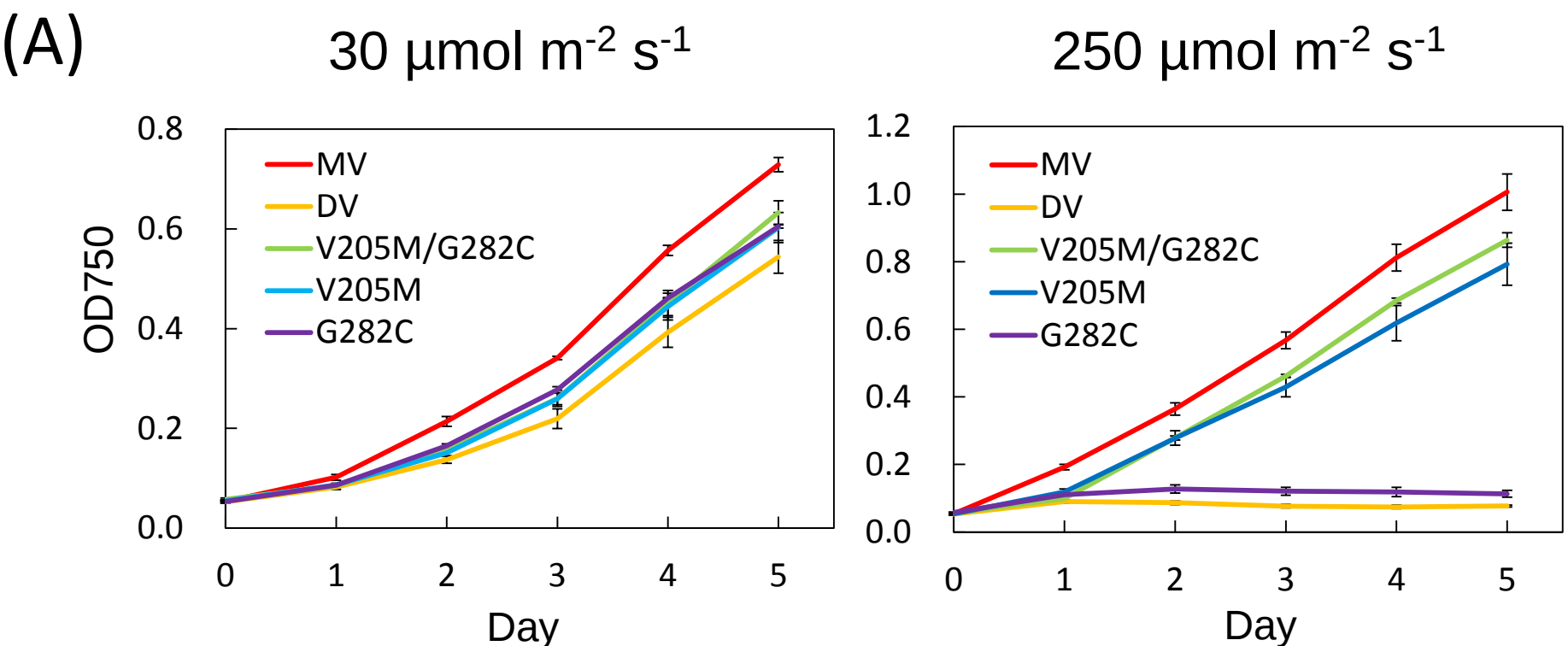


Fig. 3

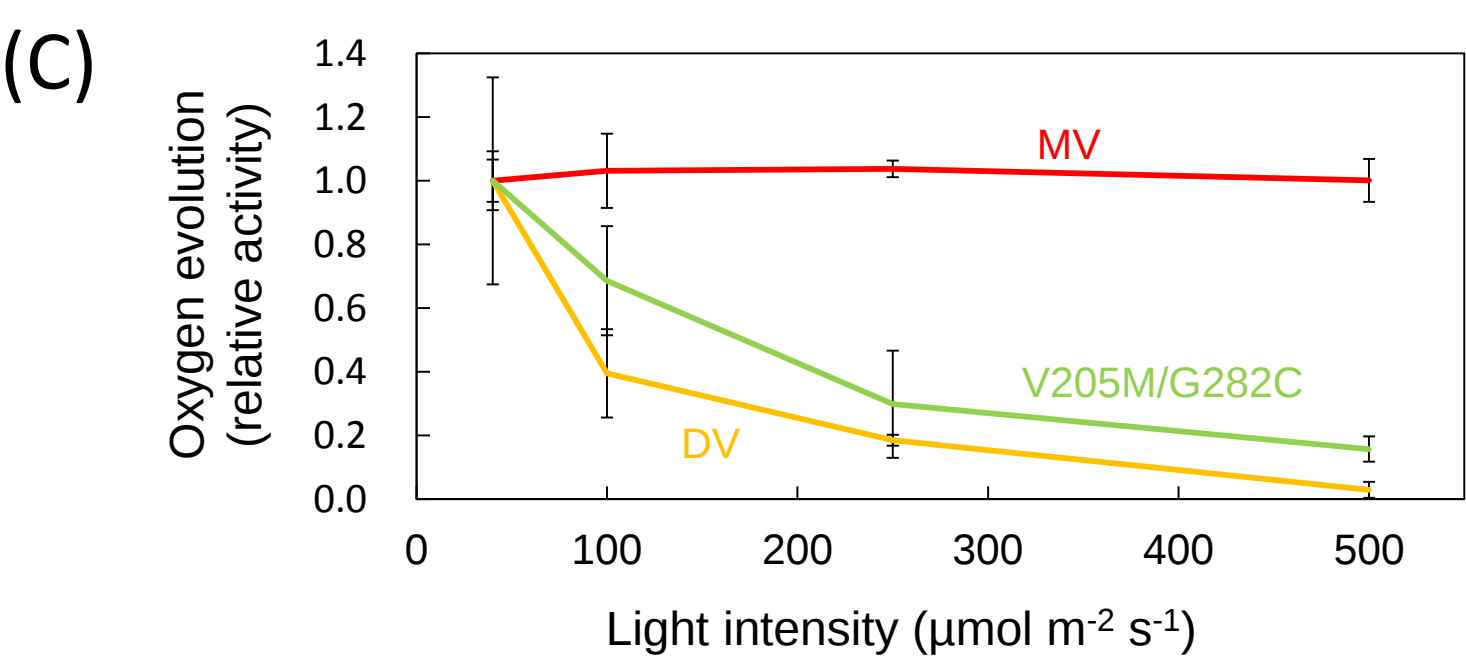
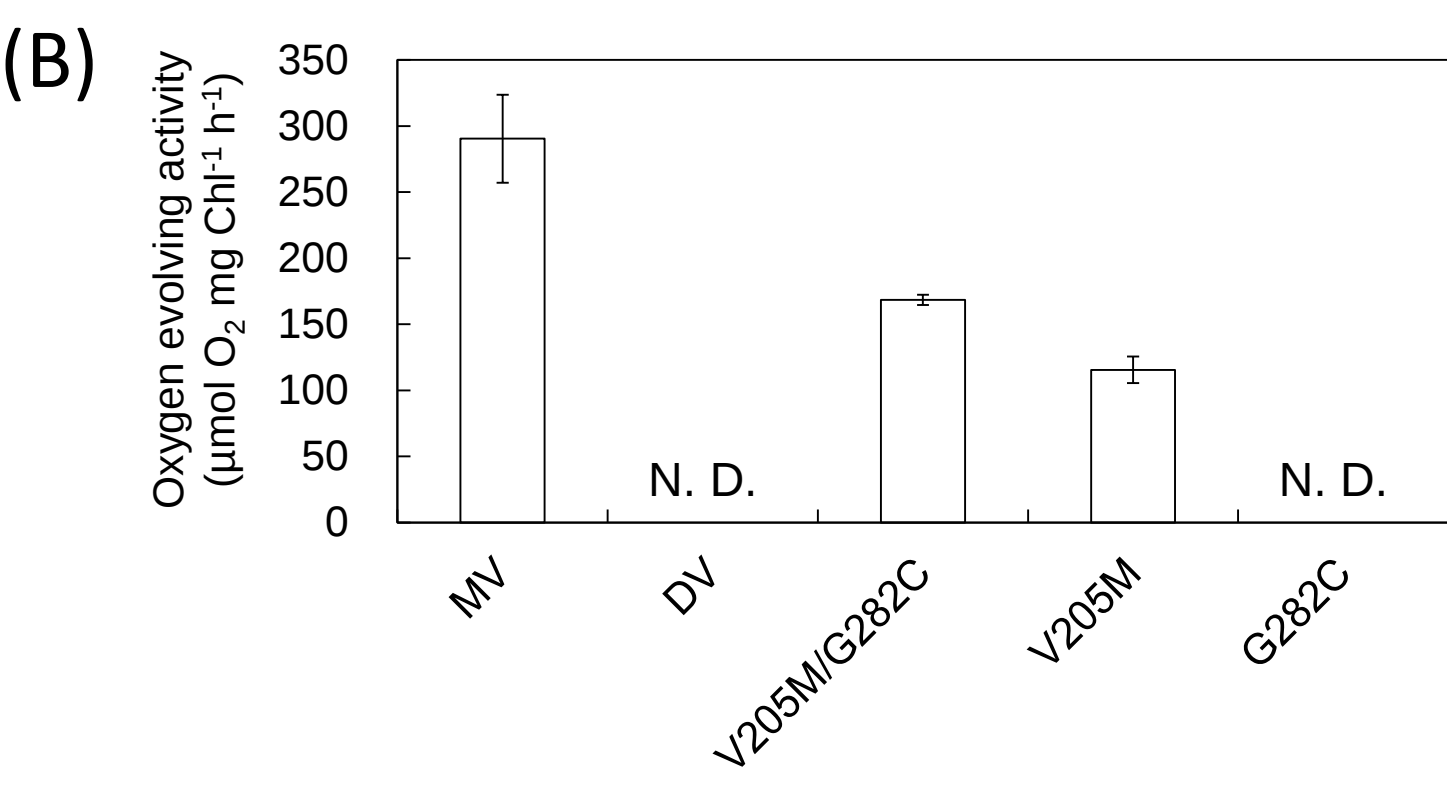
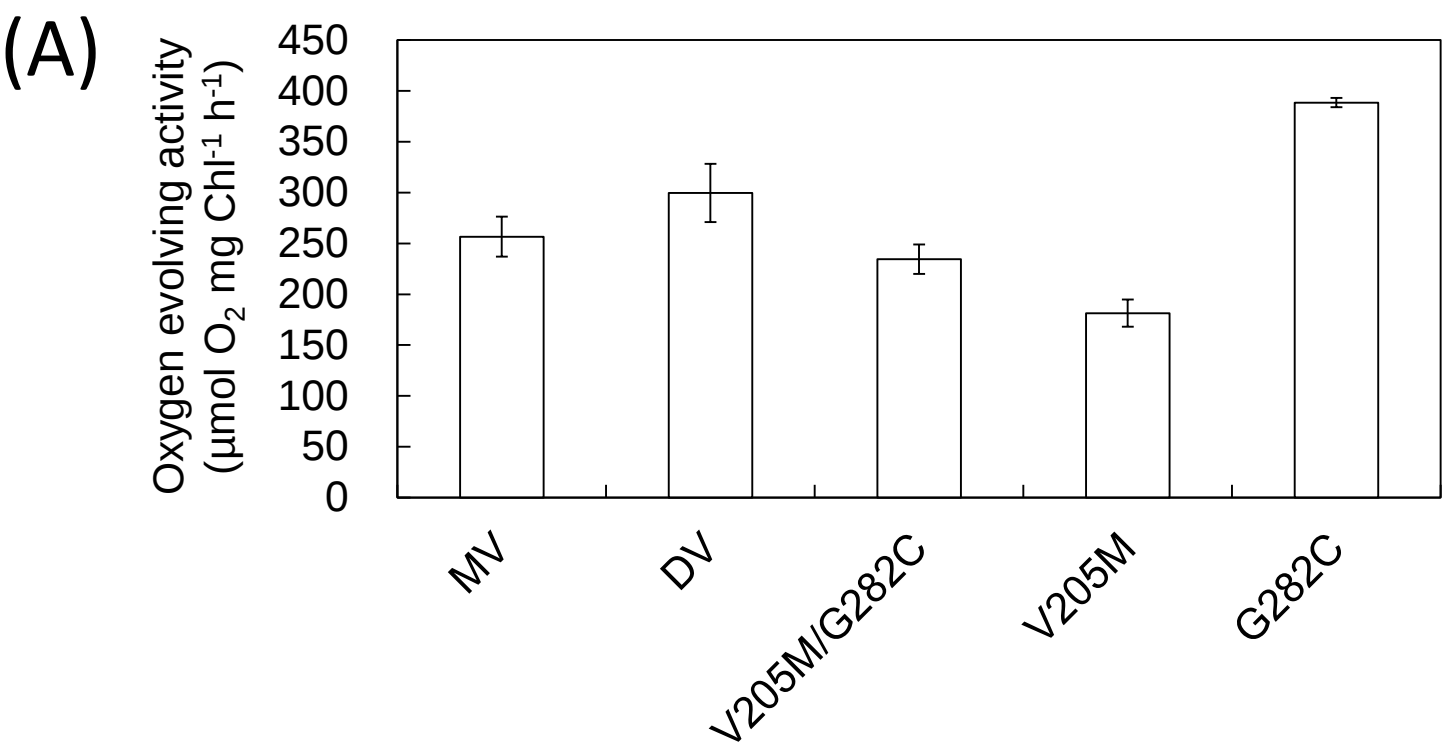


Fig. 4

Prochlorococcus

Synechococcus

Acaryochloris

Prochlorococcus

Synechococcus

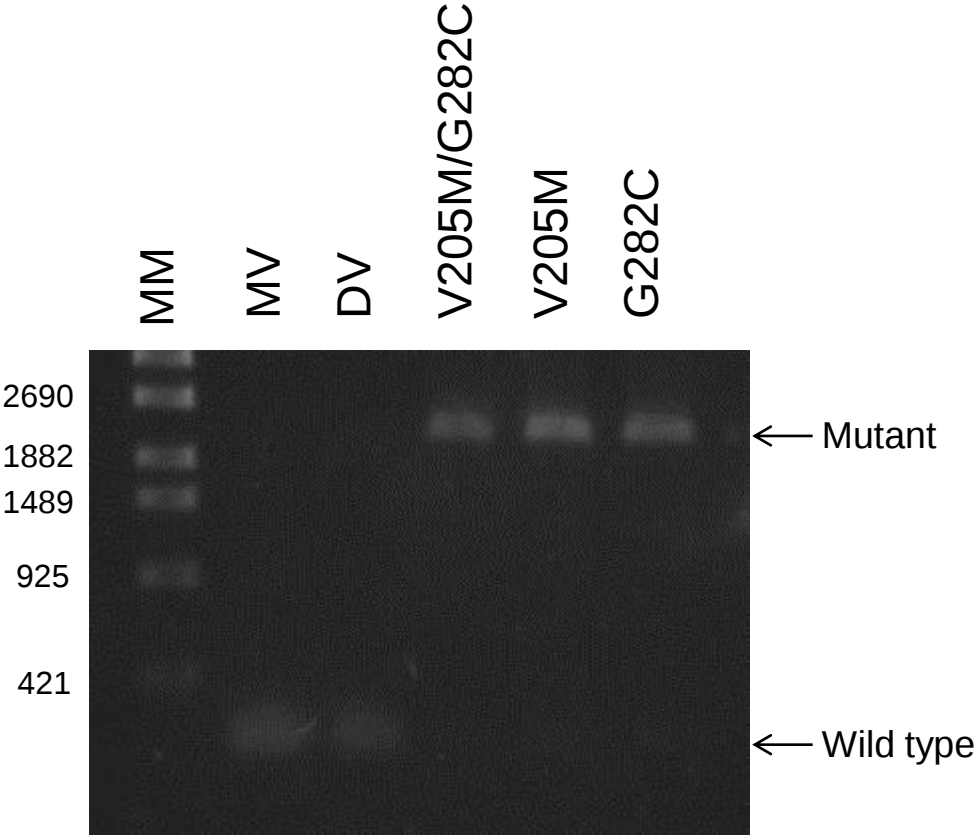
Acaryochloris

Prochlorococcus

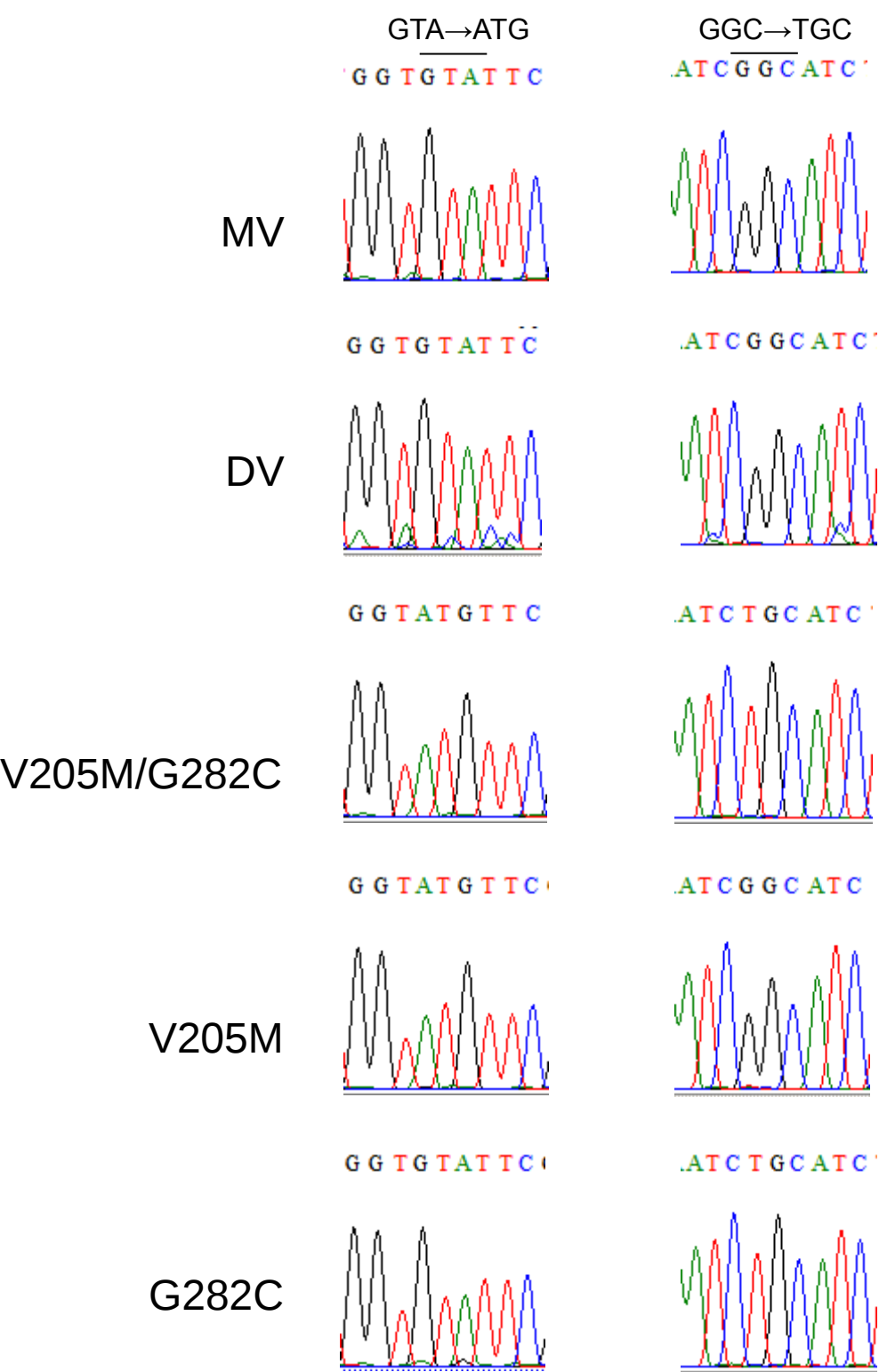
Synechococcus

Acaryochloris

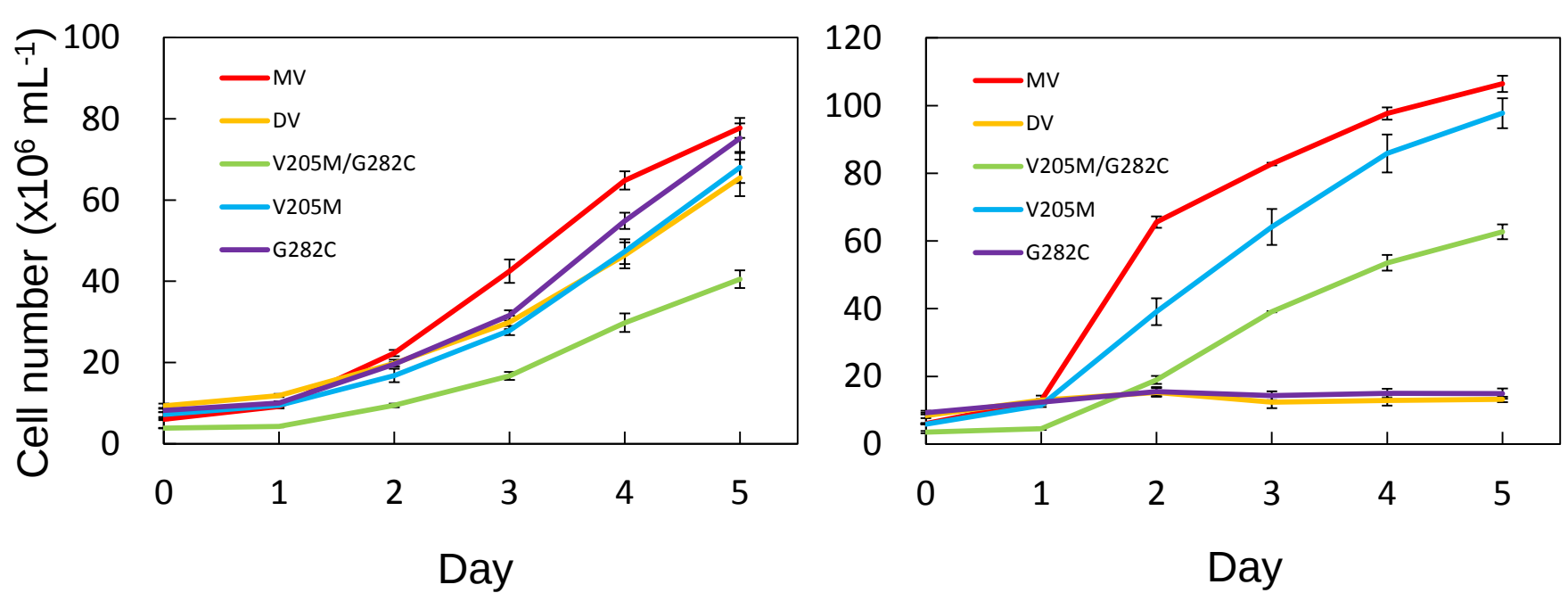
(A)



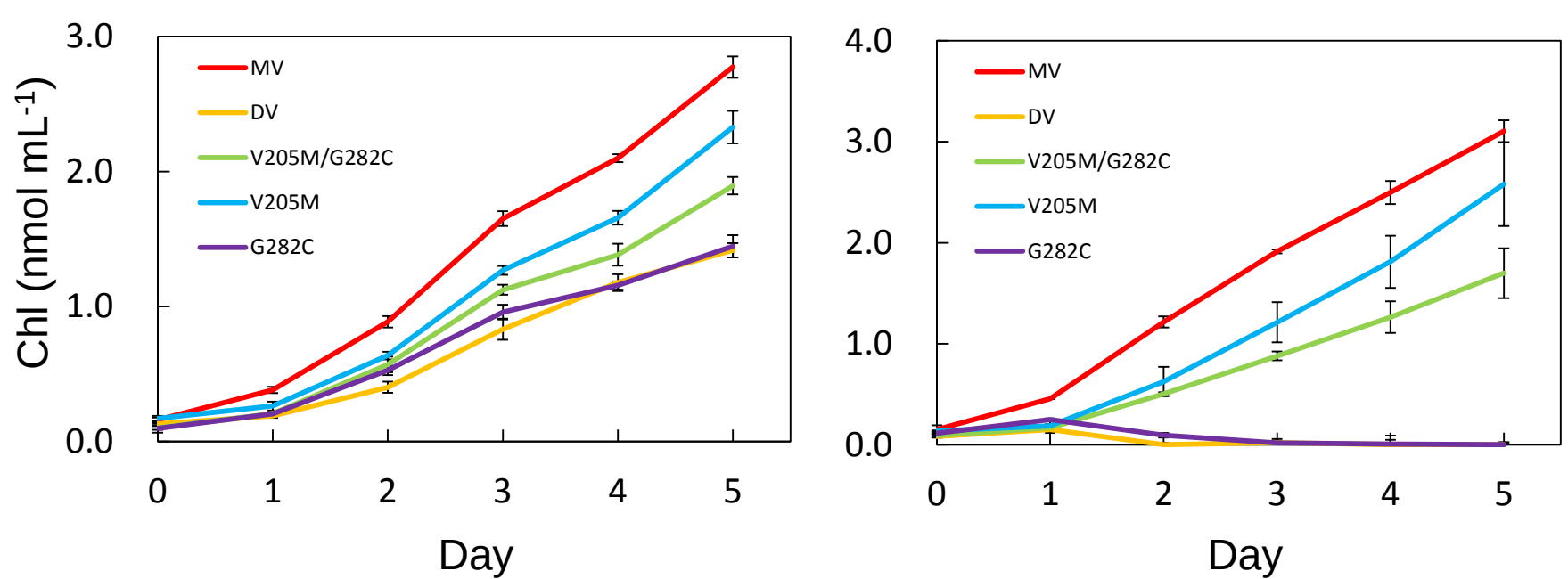
(B)

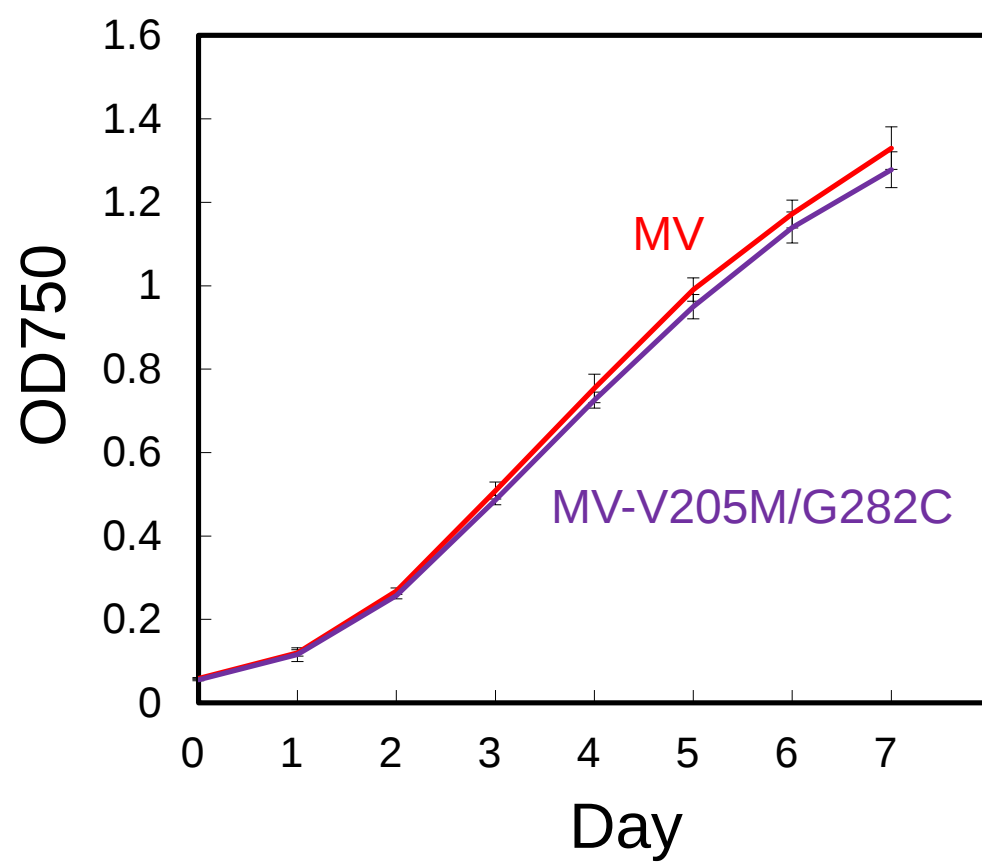


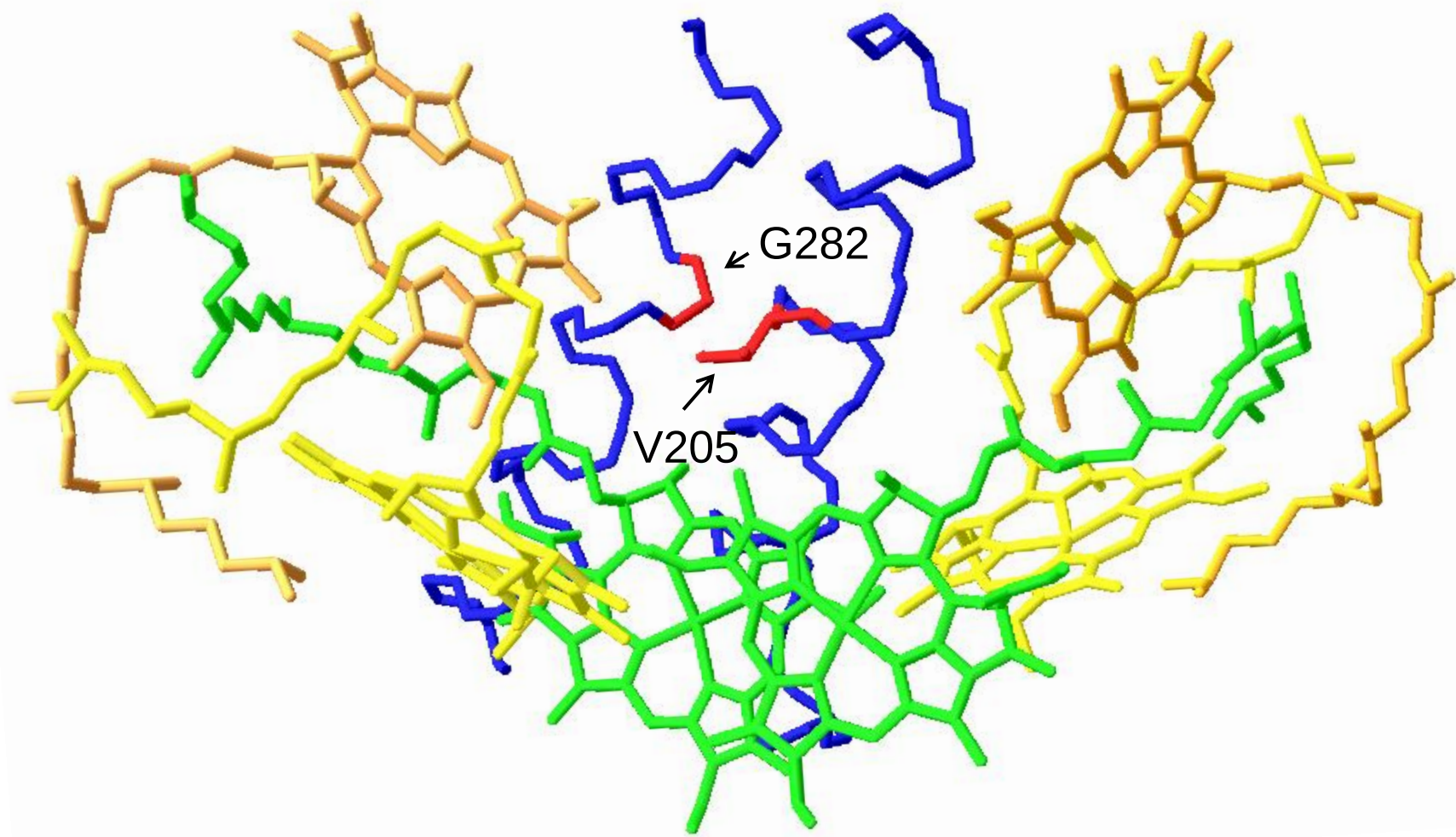
(A)



(B)



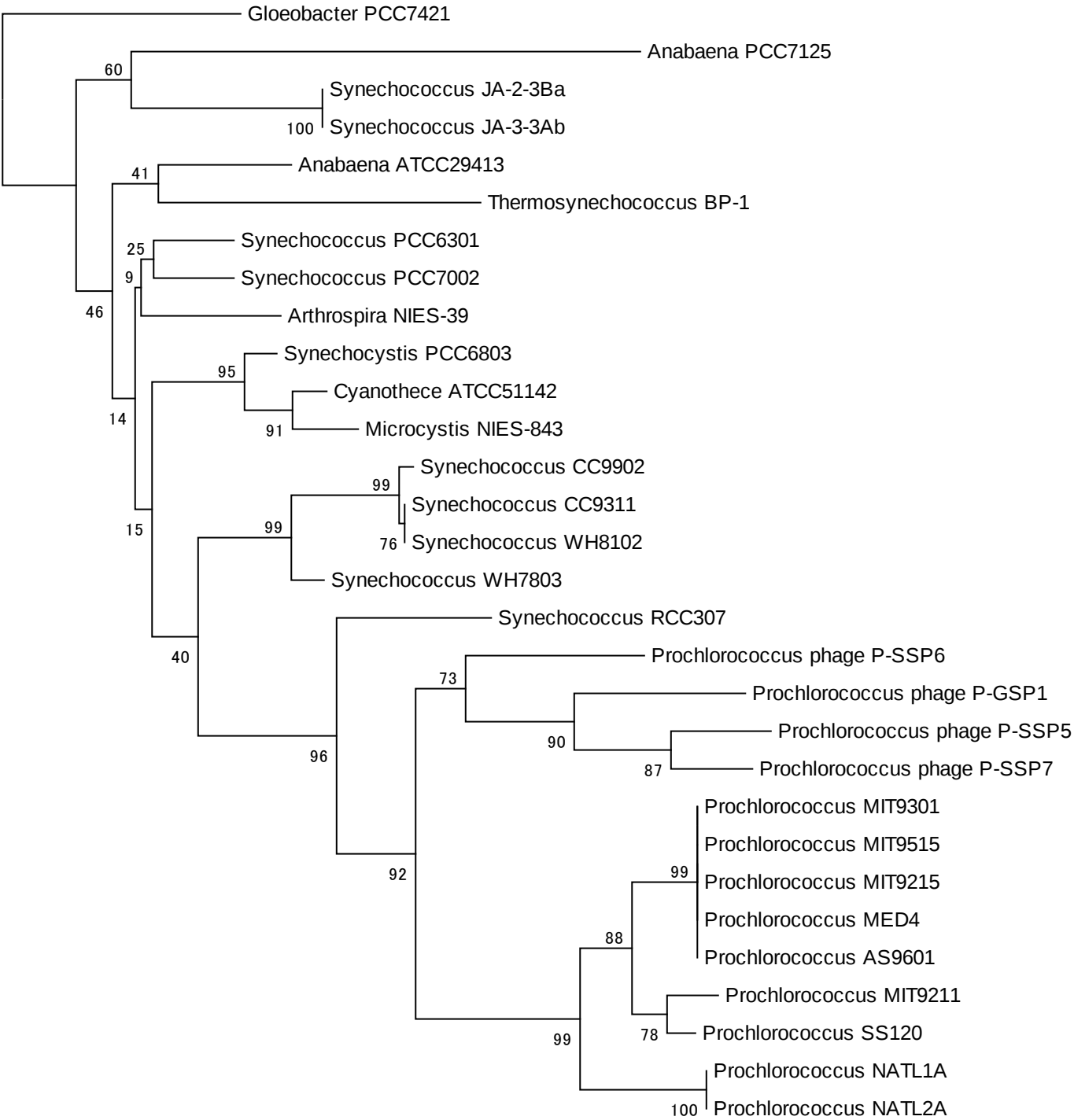




G282

V205

C-8 Ethyl group



0.01