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IDENTIFICATION OF A NOVEL VINYL REDUCTASE GENE ESSENTIAL FOR THE BIOSYNTHESIS OF MONOVINYL CHLOROPHYLL IN *SYNECHOCYSTIS* SP. PCC6803

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The vast majority of oxygenic photosynthetic organisms use monovinyl chlorophyll for their photosynthetic reactions. For the biosynthesis of this type of chlorophyll, the reduction of the 8-vinyl group that is located on the B ring of the macrocycle is essential. Previously, we identified the gene encoding 8-vinyl reductase responsible for this reaction in higher plants, and termed it *DVR*. Among the sequenced genomes of cyanobacteria, only several *Synechococcus* species contain *DVR* homologues. Therefore, it has been hypothesized that many other cyanobacteria producing monovinyl chlorophyll should contain a vinyl reductase which is unrelated to the higher-plant *DVR*. In order to identify the cyanobacterial gene that is responsible for monovinyl chlorophyll synthesis, we developed a bioinformatics tool, CCCT (Correlation Coefficient Calculation Tool), which calculates the correlation coefficient between the distributions of a certain phenotype and genes among a group of organisms. The program indicated that the distribution of a gene encoding a putative dehydrogenase-protein is best correlated with the distribution of the *DVR*-less

cyanobacteria. We subsequently knocked out the corresponding gene (*Slr1923*) in *Synechocystis* sp. PCC6803 and characterized the mutant. The knock-out mutant lost its ability to synthesize monovinyl chlorophyll and, accumulated 3,8-divinyl chlorophyll instead. We concluded that *Slr1923* encodes the vinyl reductase or a subunit essential for monovinyl chlorophyll synthesis. The function and evolution of 8-vinyl reductase genes are discussed.

Chlorophyll is an essential molecule that is utilized in photosynthetic reactions (1). Various chlorophyll species exist amongst photosynthetic organisms. Anoxygenic photosynthetic bacteria contain bacteriochlorophyll *a*, *b*, *c*, *d*, *e* and *g* (2) and oxygenic photosynthetic organisms such as cyanobacteria, algae and land plants produce chlorophyll *a*, *b*, *c*, *d* and 3,8 divinyl-chlorophyll *a*¹ and *b* (Fig. 1)(3, 4). Among them, the biosynthesis of chlorophyll *a*, *b*, *c*, *d* and all bacteriochlorophyll species requires the reduction of the 8-vinyl group (2). All of these chlorophyll molecules have a common backbone structure. Modification of the side chains on the

common backbone structure gives rise to the diversity of chlorophylls (5,6). Among the variety of chlorophyll species, bacteriochlorophyll *a* and monovinyl chlorophyll *a* (Fig. 1) are most commonly used for the photochemistry in photosynthetic organisms. The exceptions to this are a group of marine cyanobacteria, *Prochlorococcus* species (7) and a cyanobacterial symbiont, *Acaryochloris marina* (8), which use 3,8-divinyl chlorophyll *a* (Fig. 1) and chlorophyll *d* for their photochemistry, respectively. It is not clear why the majority of photosynthetic organisms prefer monovinyl chlorophylls instead of divinyl chlorophyll species. Akimoto et al. (9) recently suggested that the replacement of monovinyl chlorophylls by 3,8-divinyl chlorophylls in the *dvr* mutant of *Arabidopsis thaliana* significantly changed the antenna system and thus affected energy and electron transfer. In addition, our group also observed that the *dvr* mutant is highly susceptible to strong illumination (10). There may be some (yet unknown) advantage for electron and/or energy transfer in the reduction of the 8-vinyl to an 8-ethyl group.

For the synthesis of monovinyl chlorophyll, the reduction of the 8-vinyl group on the pyrrole ring B of 3,8-divinyl chlorophyllide (or possibly 3,8-divinyl protochlorophyllide) is prerequisite. In our previous study, we identified a gene encoding 8-vinyl reductase which is responsible for the synthesis of monovinyl chlorophyll from *Arabidopsis* and termed the gene *DVR* (10). Homologues of the *DVR* gene were found in the genomes of higher plants, green algae and Prasinophytes. In addition, most but not all

green sulfur bacteria and some purple bacteria have *DVR* homologues that were termed *bciA* (19). Interestingly, there were no homologues identified in the genome of a red alga, *Cyanidioschyzon merolae*. Homologues were also found in five *Synechococcus* species but were not identified in any other cyanobacteria. Since *Cyanidioschyzon merolae* and the majority of cyanobacteria which lack *DVR* homologues synthesize monovinyl chlorophyll, it has been hypothesized that an unidentified 8-vinyl reductase is present in these organisms.

In order to identify the second *DVR* gene, we first searched for genes exclusive to *Cyanidioschyzon merolae* and cyanobacteria which do not possess *DVR* homologues but synthesize monovinyl chlorophyll *a*. For this purpose, we developed a bioinformatics tool, “Correlation Coefficient Calculation Tool (CCCT)” to select the candidates of the cyanobacterial genes encoding the hypothetical 8-vinyl reductase. We subsequently carried out a molecular genetic analysis and finally identified the gene in cyanobacteria which is involved in the 8-vinyl reductase activity. The function of the gene product and its evolutionary history are discussed.

Experimental Procedures

Materials and growth conditions- *Synechocystis* sp. PCC6803 was grown at 30°C in BG-11 medium (11) in liquid and solid (0.5% agar) media under continuous illumination with normal light (50 $\mu\text{mol photons/m}^2\text{s}$, white fluorescence lamp) or high light (1000 μmol

photons/m²s).

Pigment analysis- Pigments were extracted with 80% acetone and analyzed by high-performance liquid chromatography (HPLC) with photodiode array detection (SPD-M20A, Shimadzu, Kyoto, Japan) on a reversed phase C18 column (150x6 mm, Shim-pack CLC-ODS, Shimadzu) using the solvent (methanol:ethyl acetate=2:1 (v:v)) at the flow rate of 0.8 ml/min (12).

Spectrometric measurements- Absorption spectra of cells were measured by using an opal diffuser at room temperatures with a Hitachi U-3310 spectrophotometer (Hitachi, Tokyo, Japan).

Electron microscopy- Wild type and mutant *Synechocystis* sp. PCC6803 cells were grown under normal light conditions. The cells were harvested by centrifugation and embedded in 3% of low melting agar. The agar containing cells were initially fixed in 2.5% glutaraldehyde in 0.1 M of cacodylate buffer (pH 7.4). Agar embedded specimens were postfixed in 1% OsO₄ in 0.1 M of cacodylate buffer and were dehydrated in a graded ethanol series. Dehydrated specimens were subsequently embedded in an epon resin mixture (TAAB Epon 812, TAAB Laboratories Equipment Ltd, Berkshire, UK) (13). Ultra-thin sections were mounted on 200-mesh size grids. Specimens were stained for 20 min with 2% (w/v) aqueous uranyl acetate and briefly with a 0.2 g/L solution of lead citrate. Specimens were viewed with a JEOL 1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 60 kV.

Gene cloning from *Synechocystis* sp. PCC 6803- To inactivate the putative 8-vinyl reductase gene

of *Synechocystis* sp. PCC6803, the *Slr1923* gene was amplified by PCR using primers 5'-GCCCAGTGGACAGGCATTGT-3' and 5'-ATGGGTAAACGTTACCGCCTT-3'. The PCR product was cloned into the pGEM T-easy vector (Promega). The kanamycin resistance gene from *Escherichia coli* was inserted into the *Sma*I site that is present in the inserted DNA fragment.

Transformation and segregation analysis of *Synechocystis* sp. PCC 6803- Transformation of *Synechocystis* sp. PCC6803 was carried out according to the methods described previously (14). Transformants were propagated on BG-11 agar plates containing 20 µg/ml of kanamycin. For the promotion of segregation, transformants were incubated under dim light (1 µmol photons/m²s) with 5 mM glucose supplementation in agar medium. The segregation state of the transformants was analyzed by PCR.

Sequence retrieval and Blast analysis- Sequences were obtained either from the GenBank database, or from Joint Genome Institute:

(http://genome.jgi-psf.org/mic_home.html). The stand-alone BLAST program (Ver. 2.2.10) (15) was obtained from NCBI and default parameters were used. Mathematica for Mac OS X, Ver. 6.0.1 (Wolfram Research, Inc. USA) was used to control the BLAST program and to calculate the correlation coefficient as described in the results section. We will freely distribute the program to calculate the correlation coefficient upon request.

Phylogenetic analysis- The deduced amino acid sequences of *DVR* and *Slr1923* homologues

were aligned using the CLUSTALW software (16) with Gonnet residue weights. A neighbor joining tree (17) was constructed on the basis of the sequence alignment with the CLUSTALW software.

RESULTS

Basic characterization of 8-vinyl reductase in Synechocystis sp. PCC 6803- Prior to the start of our bioinformatics approach to identify 8-vinyl reductase in *Synechocystis* sp. PCC 6803, we examined whether this organism possesses specific activities for the reduction of the 8-vinyl group of the tetrapyrrole. Thylakoid membranes of wild type cells were purified and solubilized by Triton X-100. 3,8-divinyl chlorophyllide *a* was incubated with the solubilized membranes and the formation of monovinyl chlorophyllide *a* was monitored by HPLC after a 15-min incubation. When NADPH was added to the reaction mixture, the formation of monovinyl chlorophyllide *a* was observed (data not shown). Without the addition of NADPH, only 3,8-vinyl chlorophyllide *a* was detected (data not shown). This experiment provided evidence which confirmed that 8-vinyl reductase is present in this organism and its activity is NADPH dependent. Thus, we initiated the following bioinformatics approach to identify the gene encoding the second 8-vinyl reductase.

Selection of possible 8-vinyl reductase genes from the cyanobacterial genome- For the following bioinformatics approach, a few assumptions were made: (1) All cyanobacteria producing monovinyl chlorophyll should have a

specific enzyme(s) that reduces the 8-vinyl group of the tetrapyrrole. (2) *Prochlorococcus* species lack 8-vinyl reductase, because they do not produce monovinyl chlorophyll. (3) There are only two types of 8-vinyl reductase in cyanobacteria. The last assumption was made in order to make our bioinformatics approach possible. Without this assumption, the list of the candidate genes for those encoding 8-vinyl reductase would become too long for the subsequent reverse-genetics approach. We should note that 5 out of 16 *Synechococcus* species whose genomes have been sequenced contain a *DVR* homologue. Therefore, it was not possible for us to accurately predict whether *Synechococcus* species have the second 8-vinyl reductase or not. Thus, we excluded those 5 species from the bioinformatics analysis. Taken together, if our assumption is correct, the second 8-vinyl reductase should exist in all cyanobacteria that produce monovinyl chlorophyll *a* except for the 5 *Synechococcus* species, and it should be absent from *Prochlorococcus* species. For the bioinformatics analysis, the cyanobacteria that were expected to have the second 8-vinyl reductase were placed into the “plus” group for bioinformatics analysis and the *Prochlorococcus* species were placed into the “minus” group. The second 8-vinyl reductase should be absent from this “minus” group. A red alga, *Cyanidioschyzon merolae*, was also included into the “plus” group, since its sequenced genome does not contain a *DVR* homologue. The addition of a eukaryotic organism to the analysis considerably increases the amount of information on the distribution of

each gene.

In general, attempts to identify orthologues by similarity searches using tools such as BLAST (15) face a common problem in determining the threshold of similarity with which orthologues are selected. If only genes showing very high similarity to the query sequence are selected as orthologues, there is a high probability for true orthologues to go undetected within the search. On the contrary, if genes showing lower similarity to the query sequence are selected as orthologues, false genes might be identified as orthologues. In order to avoid this problem, CCCT ranked possible orthologues by calculating the correlation coefficient between the ability of monovinyl chlorophyll synthesis and the E value of each gene to the query sequence. Fig. 2 shows an example of CCCT analysis. If the query sequence shows high similarity (low E value) to one of the genes that is found only in the genomes of the “plus” group, CCCT gives higher values (correlation coefficient) closer to 1. In contrast, when another sequence shows high similarity to a gene found within the genomes of the “minus” group, the program generates a lower value that is closer to -180. With this strategy, both the pattern of gene distribution and the phenotype can be compared and scored.

In this study, all ORFs from the *Synechocystis* sp. PCC 6803 genome were used as a query sequence, and BLAST searches were performed against each genome from 32 organisms that were categorized either in the “plus” or “minus” group (see Fig. 2 for the list of organisms). For each genome, an ORF

exhibiting the lowest E value to each query sequence was selected, and its value was used for the calculation of the correlation coefficient. Finally, all ORFs were ranked according to the order of higher correlation coefficient.

Figs 2B, C and D show the E value profiles of the candidate proteins that were identified through our CCCT analysis. The highest correlation coefficient was found in *Slr0335* ($r=0.998$) which encodes a phycobilisome LCM core-membrane linker polypeptide. The second and third highest were *Slr1923* (hypothetical protein, $r=0.993$) and *Sll0757* (amidophosphoribosyltransferase, $r=0.957$), respectively. As shown in Figs. 2B and C, the distribution pattern of two genes, *Slr1923* and *Slr0335*, matched our categorization pattern of the “plus” and “minus” groups. The distribution of the third gene, *Sll0750*, also matched our categorization pattern except for red algae “*Cyanidioschyzon merolae*” (Fig. 2D). *Slr0335* was excluded from further analysis because the gene encodes a known component of phycobilisome. The *Slr1923* gene has sequence similarity to the F420 reducing hydrogenase (FRH) β -subunit of *Methanobacterium thermoautotrophicum* (18). Although the reaction catalyzed by FRH is not very similar to that presumed for the reaction by 8-vinyl reductase, we thought it was possible that *Slr1923* was involved in the reduction of the 8-vinyl group, because an oxidoreductase family can potentially catalyze a wide range of reactions. Therefore, we decided to pursue this promising gene for further functional analysis.

Disruption of the Slr1923 gene- In order to

examine whether the *Slr1923* gene product participates in the reduction of the 8-vinyl group on pyrrole ring B, we disrupted the *Slr1923* gene by homologous recombination in *Synechocystis* sp. PCC 6803 (Supplementary Fig. 1). Using an HPLC system incorporating a photo-diode array detector, we analyzed the pigment compositions of the *slr1923* mutant and wild-type cells (Fig. 3). In the mutant cells, a chromatographic peak corresponding to the monovinyl chlorophyll (8.56 min, peak 3-2) disappeared. Instead, another peak appeared at an earlier elution time (8.44 min, peak 3-1) in the mutant cells. The early peak in the mutant has the same retention time and absorption spectrum to those of 3,8-divinyl chlorophyll which accumulate in the *dvr* mutant of *Arabidopsis thaliana* (10). Furthermore, this peak provided a $[m+H]^+$ m/z value (870.5) which corresponds to the Mg-dechelated 3,8-divinyl chlorophyll *a* (3,8-divinyl pheophytin *a*). In contrast, the HPLC peak corresponding to monovinyl chlorophyll of wild type gave a $[m+H]^+$ m/z value of 872.5 which represents Mg-dechelated monovinyl chlorophyll *a* (pheophytin *a*). It is important to note that the Mg ion was removed from the chlorophyll molecules during the MS analysis. Taken together, these results clearly demonstrate that *Slr1923* encodes an essential component of 8-vinyl reductase which is required for the monovinyl chlorophyll synthesis in this organism.

The *Slr1923* gene is located in a gene cluster consisting of *Slr1923*, *Slr1924*, and *Slr1925* in this order. *Slr1924* and *Slr1925* encode carboxypeptidase and a protein involved

in cobalamin biosynthesis, respectively. It was possible that disruption of *Slr1923* affected the expression of *Slr1924* and *Slr1925*. To examine whether possible disturbance in the expression of *Slr1924* and *Slr1925* had affected the synthesis of 3-vinyl chlorophyll, we inserted the kanamycin resistance gene into *Slr1924*. We found that the *Slr1924* disruptant did not show any observable phenotype including accumulation of 3,8-divinyl chlorophyll (supplementary Fig. 2). These results indicate that the *Slr1924* protein is not involved in the reduction of the 8-vinyl group. Furthermore, the same experiment indicates that *Slr1925* is neither involved in this reaction. It was because if the accumulation of 3,8-divinyl chlorophyll was caused by the interference of the expression of *Slr1925* in the *slr1923* mutant, the disruption of the *Slr1924* gene should have resulted in accumulation of 3,8-divinyl chlorophyll, but it was not the case (see supplementary Fig. 2). Collectively, we concluded that the accumulation of 3,8-divinyl chlorophyll in the *slr1923* mutant was not the result of possible disturbance in *Slr1924* and *Slr1925* gene expression.

Phenotype of the Slr1923 knock-out mutant- Disruption of *Slr1923* not only affected chlorophyll synthesis but also carotenoid synthesis. With respect to the wild type, the ratio of zeaxanthin to chlorophyll increased two-fold, however, the β -carotene-to-chlorophyll ratio remained unchanged in the mutant cells. As mentioned in the discussion section, it is possible that the increase in the zeaxanthin contents is induced by the light stresses.

Electron microscopy showed that the average size of wild type cells was about $1.33 \pm 0.04 \mu\text{m}$ ($n=4$), whereas, the *slr1923* mutant cells was about $1.15 \pm 0.07 \mu\text{m}$ ($n=4$) (Fig. 4). With respect to wild type, the number of thylakoid membranes per cell was greatly reduced in the mutant cells, and they also exhibited a wider space between thylakoid membranes. These morphological changes in the mutant cells may also indicate that they are experiencing stressful conditions. Part of the stress could be that the 3,8-divinyl chlorophyll does not bind well to the proteins and is partly present in free form, which would be highly phototoxic.

In our previous paper, we reported that the *dvr* mutant of *Arabidopsis thaliana*, whose monovinyl chlorophyll was replaced by 3,8-divinyl chlorophyll, was completely bleached within a day under exposure to high light conditions (10). We were therefore intended to determine whether cyanobacteria containing 3,8-divinyl chlorophyll also exhibit a similar response to strong illumination. Wild-type and mutant cells were initially cultured in liquid medium under normal light conditions ($50 \mu\text{mol photons/m}^2\text{s}$) and were subsequently transferred to high light conditions ($1000 \mu\text{mol photons/m}^2\text{s}$). Wild-type cyanobacteria turned yellow slightly but were able to survive exposure to high light illumination (Fig. 5). In contrast, *slr1923* mutant cells were completely bleached after 1 day of high light treatment (Fig. 5). These convincing results may indicate that the replacement of monovinyl chlorophyll with 3,8-divinyl chlorophyll induces a similar response through

an analogous mechanism between higher plants and cyanobacteria.

The growth rate of the *slr1923* mutant was indistinguishable from that of the wild type cells under low light conditions ($10 \mu\text{mol photons/m}^2\text{s}$), and it was slightly lower than the wild type cells under standard light conditions ($40 \mu\text{mol photons/m}^2\text{s}$) (Fig. 6). Under high light conditions ($150 \mu\text{mol photons/m}^2\text{s}$), the growth of *slr1923* mutant was significantly retarded. These results also showed that the mutant accumulating 3,8-divinyl chlorophyll was sensitive to high light irradiation.

Spectral analysis of the mutant- In order to further identify the effects of the accumulation of 3,8-divinyl chlorophyll, absorbance spectra of the mutant cells were measured (Fig. 7). The absorbance peak in the *soret* region was red-shifted by 6 nm in the mutant due to the accumulation of 3,8-divinyl chlorophyll. Absorption corresponding to chlorophyll (around 440 nm and 660 nm), carotenoids (around 490 nm) and phycobiliproteins (around 630 nm) was observed in both wild type and mutant cells. With respect to wild type, absorption corresponding to chlorophyll decreased in the mutant. However, absorption corresponding to phycobilisomes increased in the mutant. These data indicate that the ratio of phycobiliproteins to chlorophyll increased in the *slr1923* mutant. Since a decrease in chlorophyll content was observed with in the *dvr* mutant of *Arabidopsis* (10), it is possible that the same phenomenon may have occurred in the *slr1923* mutant. It should be noted that phycobilin synthesis was not inhibited in the *slr1923*

knock-out mutant as judged by its absorption spectrum (Fig. 5). Considering that phycobilins are synthesized by oxidative ring opening of heme, these data indicate that the common pathway of the heme and chlorophyll biosynthesis was not significantly affected in the mutant. Instead, it is likely that the chlorophyll branch of tetrapyrrole synthesis is predominantly inhibited compared to the heme branch. A possible explanation is that chlorophyll proteins are destabilized, causing the amount of free chlorophyll or chlorophyllide to rise, which in turn feedback to reduce the flux of intermediates through the pathway.

Phylogenetic analysis of 8-vinyl reductase- We analyzed the distribution of *Slr1923* homologues in photosynthetic organisms (Fig. 8A). We could not find any *Slr1923* homologues in the *Synechococcus* species in which we found *DVR* homologues, and in *Prochlorococcus* species. Instead, we found *Slr1923* homologues in other cyanobacteria that have been deposited in the Genbank database. In addition, *Slr1923* homologues were found in higher plants, green sulfur bacteria, green filamentous bacteria and purple bacteria, and their predicted amino acid sequences are highly conserved (Supplementary Fig. 3A for the alignment of the sequences). Despite this high conservation, there is no evidence showing that these *Slr1923* homologues encode functional 8-vinyl reductase genes, with the exception for those belonging to the cyanobacterial clade. According to the public microarray database in NASC, the *Arabidopsis* homologue of *Slr1923* was predominantly expressed in the photosynthetic

organs and its expression was increased by the induction of leaf senescence in *Arabidopsis*. Interestingly, our phylogenetic analysis suggested that a red algal sequence belongs to the cyanobacterial clade.

For comparison with the phylogenetic tree of *Slr1923* homologues, we constructed a phylogenetic tree for *DVR* homologues. We found *DVR* homologues in higher plants, green algae, prasinophytes, diatoms, green sulfur bacteria, cyanobacteria (*Synechococcus* species) and purple bacteria (Fig. 8B). We expressed recombinant *DVR* proteins from *Arabidopsis* (10) and *Synechococcus* WH8102 (unpublished results) in *E. coli* and confirmed that these protein have 8-vinyl reductase activity for 3,8-divinyl chlorophyllide *a*. Thus, it would be reasonable to assume that all *DVR* homologues in the green lineage clade and the *Synechococcus* clade encode functional 8-vinyl reductase. In addition, Chew and Bryant (19) also detected the 8-vinyl reductase activity with a recombinant BciA protein from *Chlorobium tepidum*. Therefore, it is likely that all the proteins belonging the green sulfur bacterial clade are genuine 8-vinyl reductase enzymes. To the best of our knowledge, there is no current evidence which shows that proteins belonging to other clades are also functional 8-vinyl reductase enzymes, even though their predicted amino acid sequences are well conserved (Supplementary Fig. 3B for the alignment of the sequences).

DISCUSSION

Bioinformatics analysis- Three bioinformatics

strategies may be potentially utilized to aid the identification of genes that are responsible for certain cellular functions. The first strategy is based upon a motif search. Proteins that participate in specific cellular processes often have distinctive motifs, such as the Rieske center binding motif. This strategy has been proven successful when searching within an organism containing only a limited number of proteins possessing the motif of interest (e.g. (20,21)). This strategy is only applicable when the protein (or gene) of interest is predicted to have a distinctive motif. In the case of 8-vinyl reductase, it was difficult to predict whether or not this protein possesses a distinctive motif that could be identifiable through a motif search. For a second strategy which aids in the identification of target genes, one can employ a co-expression analysis (22). This type of gene expression analysis compares the expression patterns of a selected gene with the other genes from a model organism such as *Arabidopsis thaliana*. This method is powered by comparative analysis of data that has been deposited on public DNA microarray databases. This type of gene expression analysis is only applicable when a large microarray database is available for the organism of interest. In addition, it is limited to the study of genes exhibiting a predictable expression pattern. Therefore, this method was applicable to the search for the novel 8-vinyl reductase gene. The third method is based on the comparison of the gene sets between organisms. Generally speaking, a particular function of a certain organism should be provided by some specific gene products. If the correlation

between the occurrence of a particular function and a specific gene is estimated among various organisms, it facilitates the prediction of the gene that is responsible for the function of interest. Previously, we successfully identified the gene encoding chlorophyllide *a* oxygenase (an enzyme responsible for chlorophyll *b* biosynthesis) in *Prochlorococcus* species by comparing the gene sets between a chlorophyll *b*-containing organism, *Prochlorococcus marinus* and chlorophyll *b*-less *Synechococcus* species (23). However, the bioinformatics tool that was used in the aforementioned study was only applicable to the closely related organisms (23). This tool also had limitations since it cannot analyze the genomes of more than 4 organisms. In the present study, we developed a new tool, CCCT, which can compare the gene sets from an unlimited number of organisms. With this method, we successfully identified the gene involved in the reduction of the 8-vinyl group of the tetrapyrrole. Since the number of available genome sequences is rapidly increasing, our new method may be applied for a wider range of genes of interest.

Function of Slr1923- Disruption of *Slr1923* resulted in the replacement of monovinyl chlorophyll by 3,8-divinyl chlorophyll. These data indicated that *Slr1923* is involved in the reduction of the 8-vinyl group. However, when recombinant *Slr1923* protein was expressed in *E. coli*, we were not able to detect any enzymatic activity (data not shown). It is possible that the *Slr1923* gene product did not form a proper conformation due to the heterologous gene expression system of *E. coli* and was therefore

rendered unable to exhibit enzymatic activity. It is also possible that additional proteins are required for its catalytic activity. *Slr1923* has sequence similarity to the gene encoding the β subunit of FRH in *Methanobacterium thermoautotrophicum* (24). Three heteromeric FRH subunits (α , β and γ) form an $(\alpha\beta\gamma)_8$ complex in this organism. The β subunit was expected to bind the substrate molecule and has a catalytic function. The α and γ subunits participate in the electron transfer from a hydrogen molecule to the β subunit (18). The genome of *Synechocystis* sp. PCC6803 contains homologous genes to α (*Sll1226*) and γ (*Sll1224*) subunits, both of which form a gene cluster. We disrupted *Sll1224* and *Sll1226* by site directed mutagenesis, however, 3,8-divinyl chlorophyll did not accumulate in the both mutant (Supplementary Fig. 4). These data indicate that *Sll1224* and *sll1226* are not involved in the reduction of the 8-vinyl group. Therefore, we concluded that the *Slr1923* gene product does not form a complex similar to FRH. These results are plausible if we consider the predicted biochemical properties of the *Slr1923* protein with that of FRH. In the reaction of FRH, the α and γ subunits of FRH receive two electrons from an unknown electron donor one by one, and transfer them to the FAD cofactor in the β subunit one by one (25). Then the β subunit reduces the substrate by transferring two electrons at a time from FADH₂. In contrast, we found that a two-electron donor, NADPH, is involved in the reaction of 8-vinyl reductase. Therefore, it is likely that two electrons are transferred to the FAD molecule that possibly

resides in the *Slr1923* protein, and then the electrons are transferred to the 8-vinyl group of the tetrapyrrole simultaneously. If this assumption is correct, the accessory proteins that play the same roles as the α and γ subunits of FRH would not be necessary for the 8-vinyl reduction. Further studies are necessary to clarify the function of the *Slr1923* gene product.

Homologues of Slr1923-We found the homologues of *Slr1923* in various organisms including eukaryotes, cyanobacteria and photosynthetic bacteria. Interestingly, a highly homologous gene exists in the *Arabidopsis* genome (*At1G04620*). The predicted amino acid sequence of the *At1G04620* gene product shows 53% amino acid identity and 68% similarity with that of the *Slr1923* gene product. Besides, *At1G04620* is predicted to encode a transit peptide for chloroplast targeting. Based on these collective observations, it is tempting to speculate that *At1G04620* is involved in the reduction of the 8-vinyl group in *Arabidopsis*. However, in the null mutant of the *Arabidopsis dvr* gene, all detectable monovinyl chlorophyll was replaced by 3,8-divinyl chlorophyll (26). This observation suggests that the DVR protein is the sole 8-vinyl reductase in *Arabidopsis*. There are two possible explanations for this discrepancy. One explanation is that the *At1G04620* product could function as 8-vinyl reductase, but the contribution of this enzyme to the synthesis of monovinyl chlorophyll may be very limited. As a result, the *dvr* mutant predominantly accumulated 3,8-divinyl chlorophyll. Another explanation is that the *At1G04620* locus does not encode 8-vinyl

reductase in *Arabidopsis* despite the high level of sequence similarity to *Slr1923* (see Supplementary Fig. 3). At present, we cannot conclude whether the *At1G04620* locus encodes a functional 8-vinyl reductase or not in *Arabidopsis*. Similarly, it is not clear whether the genes (or proteins) belonging to the higher-plant clade encode 8-vinyl reductase (Fig. 8).

It is noteworthy that the *Slr1923* homologue in *Cyanidioschyzon merolae* was categorized into the cyanobacterial clade (Fig. 8A). Since this organism does not contain a *DVR* homologue, and since the *Slr1923* homologue of this organism is very similar to those of cyanobacteria (see Supplementary Fig. 3), it would be reasonable to assume that the *Slr1923* of *Cyanidioschyzon merolae* encodes a functional 8-vinyl reductase. The classification of the *Slr1923* of *Cyanidioschyzon merolae* in the cyanobacterial clade is in contrast to that of the other eukaryotic homologues in a distinct higher-plant clade (Fig. 8A). This result indicates that the *Slr1923* homologue of *Cyanidioschyzon merolae* has a different evolutionary history from those of the other eukaryotic organisms. It is possible that the cyanobacterial genes for *Slr1923*-related 8-vinyl reductase are horizontally transferred from the genome of *Cyanidioschyzon merolae* or vice versa.

Due to a lack of functional experimental evidence, it is also unclear whether the *Slr1923* homologues in photosynthetic bacteria encode functional 8-vinyl reductase. The function of *DVR* homologues is also not known for purple bacteria and diatoms. In order to discuss the

evolutionary history of 8-vinyl reductase genes, we summarized the distribution of *DVR* and *Slr1923* homologues in representative photosynthetic bacteria and cyanobacteria in Table 1. In this table, a homologue belonging to a phylogenetical clade in which at least one gene was shown to encode the functional enzyme was indicted as “+”. Homologues belonging to a clade in which functionality has not been experimentally confirmed is indicated as “±”. Lastly, organisms which lack a homologue are denoted by “-”.

Two extreme hypotheses were developed to discuss the evolutionary history of 8-vinyl reductase genes. In the first hypothesis, we assumed that all *DVR* (*BciA*) and *Slr1923* homologues indicated in Fig. 8 encode functional 8-vinyl reductase. In other words, homologues of all “+” and “±” organisms encode the functional enzyme. This assumption led us to conclude that only *Roseiflexus* species lack both *BciA* and *Slr1923* homologues among the organisms in which the 8-vinyl group is reduced. Thus, upon this assumption, we predict that there is an unknown type of 8-vinyl reductase in the group of *Roseiflexus*. Upon the same assumption, we can also conclude that in certain green sulfur bacteria including *Chlorobium phaeobacteroides* DSM266, a *BciA* homologue is replaced by an *Slr1923* homologue. *Chlorobium phaeobacteroides* DSM266 may have acquired the *Slr1923* homologue by a horizontal gene transfer from purple bacteria or cyanobacteria.

Alternatively, we hypothesized that only *DVR* in the cyanobacterial and eukaryotic clades

and *Slr1923* in the cyanobacterial clade encode the functional 8-vinyl reductase. In this second hypothesis, we need additional 8-vinyl reductase genes in order to explain the distribution of the ability to reduce the 8-vinyl group. One possible candidate for the third 8-vinyl reductase is the *BchJ* encoded protein which was suggested to encode 8-vinyl reductase in *Rhodobacter sphaeroides* (27). However, it is probably not the case, because the *bchJ* disruptant of *Rhodobacter sphaeroides* still produced certain amounts of 8-vinyl BChl *a* (27). In addition, Chew and Bryant did not detect 8-vinyl reductase activity with the recombinant BchJ protein of *Chlorobium tepidum* (19). Therefore, we should assume a yet unknown protein for the second hypothesis. Similar to the first hypothesis, horizontal gene transfer could explain the absence of *Slr1923* or *BciA* homologues in *Roseiflexus* species and in *Chlorobium phaeobaceroideis*. Taken together, both hypotheses with opposite assumptions indicate the presence of additional 8-vinyl reductase genes and horizontal gene transfer.

The mosaic distribution pattern of 8-vinyl reductase genes among photosynthetic organisms is not similar to most of the currently identified genes that are involved in the chlorophyll biosynthesis (2). Among the genes for chlorophyll biosynthesis, those encoding glutamyl-tRNA reductase, glutamate-1-semialdehyde aminotransferase, 5-aminolevulinate synthase, coproporphyrinogen III oxidase, Mg-protoporphyrin IX monomethyl ester cyclase, and protochlorophyllide oxidoreductase are not ubiquitously found in

photosynthetic organisms. The genes for 5-aminolevulinate synthase are found in some purple bacteria, while in other photosynthetic organisms, the genes for glutamyl-tRNA reductase and glutamate-1-semialdehyde aminotransferase are present. A rare exception is *Euglena gracilis*. This organism seems to contain all three enzymes (28), and this unique distribution may reflect the fact that its genome is a hybrid of photoautotrophic and heterotrophic genomes. (29). In the case of coproporphyrinogen III oxidase, Mg-protoporphyrin IX monomethyl ester cyclase, and protochlorophyllide oxidoreductase, both aerobic and anaerobic types of enzymes exist in photosynthetic organisms. One or both types of enzymes are used in an organism in order to cope with either the aerobic or anaerobic environments that they must to survive in. The distribution of these enzymes among photosynthetic organisms does not seem to contradict the evolutionary tree of organisms (30,31). Thus, it would be interesting to learn why the distribution of 8-vinyl reductase genes is so unique among the genes which encode the enzymes in chlorophyll biosynthesis. One possible explanation is that the *DVR*-type 8-vinyl reductase has just evolved recently probably in eukaryotic organisms, and then, the gene is currently spreading among photosynthetic organisms by dominating the pre-existing *Slr1923* genes. This process might be traceable by evaluating the distribution of 8-vinyl reductase genes in cyanobacteria. The phylogenetic tree of *Slr1923* homologues strongly suggests that an ancestor of

cyanobacteria had the *Slr1923*-type 8-vinyl reductase (Fig. 8A). In lineages of *Synechococcus* species, the *Slr1923* homologues might have been driven away by *DVR* homologues, if a *DVR*-type enzyme was superior to the *Slr1923* type for unknown reasons. Collectively, the genes for 8-vinyl reductase may provide an interesting model to examine the process of horizontal transfer of genes among photosynthetic organisms.

Phenotype of the cyanobacterial 8-vinyl reductase mutant- The knock-out mutant for the *Slr1923* locus accumulated a higher level of zeaxanthin. This carotenoid species protects cells from photoinhibition (32,33). The level of zeaxanthin increases by strong illumination (32). Therefore, it is possible that oxidative stress was generated in the *slr1923* mutant cells even under normal light conditions.

We also observed a reduction in cell size and the number of thylakoid membranes. These phenomena may be partly due to the stress induced by the accumulation of 3,8-divinyl chlorophyll *a*. It is consistent with the observation that heat shock stress enlarged intra-thylakoid membrane spaces in *Synechocystis* sp. PCC 6803 cells (34). Stress conditions would affect the organization of thylakoid membrane.

Akimoto et al reported that the time-resolved fluorescence spectra were significantly changed in the *dvr* mutant of *Arabidopsis* (9). Moreover, the delayed fluorescence from the photosystem II was not observed in the *dvr* mutant (9). These data suggest that both the antenna system and the electron transfer system were affected in the mutant. Further analysis of mutants producing 3,8-divinyl chlorophyll may reveal why most photosynthetic organisms utilize monovinyl-type chlorophylls for their photosynthesis.

In summary, we identified the gene encoding the 8-vinyl reductase or its essential subunit in most cyanobacteria in this study. A phylogenetic analysis of this gene led us to hypothesize that a third enzyme involved in the 8-vinyl reductase exists in photosynthetic bacteria. It also illustrates the unique distribution patterns of the genes involved in the reduction of the 8-vinyl group. Further phylogenetic analyses of the *DVR* (*BciA*) and *Slr1923* genes and functional analyses of the 8-vinyl reductase mutants may increase our understanding to the evolution of pigment biosynthesis in photosynthetic organisms.

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FOOTNOTES

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¹According to the IUPAC nomenclature, chlorophyll *a* has a vinyl group at position 3 and an ethyl group at position 8 (see Fig. 1). Accordingly, what we call 3,8-divinyl chlorophyll *a* in this paper should be termed “8-deethyl-8-vinyl chlorophyll *a*”. However, most plant physiologists commonly call this compound “3,8-divinyl chlorophyll *a*” or simply “divinyl chlorophyll *a*”. We think this nomenclature is easier to understand. Therefore, we used the common nomenclature “3,8-divinyl chlorophyll *a*” instead of the IUPAC nomenclature in this paper

The abbreviations used are: CCCT, correlation coefficient calculation tool; HPLC, high-performance liquid chromatography; FRH, F420 reducing hydrogenase.

FIGURE LEGENDS

Fig. 1. 3,8-divinyl chlorophyll *a* and monovinyl chlorophyll *a*. R denotes a phytol. The position of the 8-vinyl group is indicated by a circle on each chemical structure.

Fig. 2. Graphic representation of the results of CCCT analysis. CCCT calculated the

correlation coefficient between the two variables, that is, the presence of homologues to the query sequences (*Synechocystis* ORFs) and that of the hypothetical 8-vinyl reductase. The organisms whose sequences were used for CCCT analysis were indicated on the X axis. “+” or “-” after the label for each organism indicates the hypothetical presence or absence of the second (non-plant type) 8-vinyl reductase. A. The theoretical model shows the ideal pattern in which the hypothetical presence of the second 8-vinyl reductase (absence of plant-type DVR homologues) and the presence of homologues (which is expressed by the E value of the BLAST search) to the query sequence perfectly match with each other. B, C, D. The graphical representation of the correlation patterns for three loci that were listed at the top by CCCT analysis.

Fig. 3. Accumulation of 3,8-divinyl chlorophyll in the *slr1923* knock-out mutant. Pigments were extracted from wild type (solid line) and the *slr1923* mutant (dotted line) and analyzed by HPLC. Eluates were detected at 435 nm. Insets show the absorption spectra of chromatographic peaks corresponding to the major chlorophyll species in wild type (3-2, solid line) and the *slr1923* mutant (3-1, dotted line). 1, myxoxanthophyll; 2, zeaxanthin; 3-1, 3,8-divinyl chlorophyll *a*; 3-2, chlorophyll *a*; 4, echinenone; 5, β -carotene. Pigments are assigned based on their absorption spectra and HPLC retention time. Peak intensity was normalized by the integrated chromatographic area for chlorophyll.

Fig. 4. Alteration of the cell size and organization of thylakoid membranes. Electron microscopic images from wild type cells (A) and *slr1923* mutant cells (B). Scale bar=200 nm.

Fig. 5. Appearance of the *slr1923* mutant cells under strong illumination. Wild-type and transformed *Synechocystis* sp. PCC 6803 were cultured for 24 hour under high light conditions (1000 $\mu\text{mol photons/m}^2\text{s}$) at 23 °C. Both cultures had the same cell concentration (OD750=1.0).

Fig. 6. High light inhibition of *slr1923* mutant growth. Growth of wild type (solid line) and *slr1923* mutant (dotted line) were measured as turbidity (OD at 750 nm) every day under various light intensities. Circle, 10 $\mu\text{mol photons/m}^2\text{s}$; square, 40 $\mu\text{mol photons/m}^2\text{s}$; triangle, 150 $\mu\text{mol photons/m}^2\text{s}$. Data are the average of two or three replicates and bars denote standard deviations ($\pm\text{SD}$).

Fig. 7. Absorption spectra of the *slr1923* mutant cells. Cell absorbance spectra were obtained using an opal diffuser. Both cultures contained an identical cell concentration (OD750=1.0). Solid line, wild type; dotted line, *slr1923* mutant.

Fig. 8. Phylogenetic analyses of *Slr1923* and DVR homologues. A and B, Neighbor-joining

trees constructed with the translated sequences of *Slr1923* and *DVR* homologues, respectively.

Bootstrap values for each clade are indicated on each node. Accession numbers for the panel A are as

follows: *Anabaena variabilis* ATCC 29413,YP_324708;*Arabidopsis*

thaliana,NP_171956;*Chlamydomonas Reinhardtii*,jgi|Chlre3|106754;*Chlorobium ferrooxidans* DSM

13031,ZP_01386063;*Chlorobium limicola* DSM 245,ZP_00511199;*Chlorobium phaeobacteroides*

DSM 266,YP_910679;*Chloroflexus aggregans* DSM 9485,ZP_01515351;*Chloroflexus aurantiacus*

J-10-fl,ZP_00768515;*Crocospaera watsonii* WH 8501,ZP_00515030;*Cyanidioschyzon*

merolae,CMJ076C;*Cyanothece* sp. CCY0110,ZP_01726467;*Dinoroseobacter shibae* DFL

12,ZP_01583710;*Gloeobacter violaceus* PCC 7421,NP_923824;*Halorhodospira halophila*

SL1,YP_001003206;*Jannaschia* sp. CCS1,YP_508242;*Loktanella vestfoldensis*

SKA53,ZP_01001937;*Lyngbya* sp. PCC 8106,ZP_01620881;*Nodularia spumigena*

CCY9414,ZP_01631202;*Nostoc* sp. PCC 7120,NP_485641;*Nostoc punctiforme* PCC

73102,ZP_00109764;*Oryza sativa* (*japonica* cultivar-group),BAF14371;*Ostreococcus lucimarinus*

CCE9901,ABO94518;*Ostreococcus tauri*,jgi|Ostta4|18248;*Pelodictyon phaeoclathratiforme*

BU-1,ZP_00589210;*Populus Trichocarpa*,jgi|Poptr1_1|230014;*Prosthecochloris aestuarii* DSM

271,ZP_00591792;*Rhodopseudomonas palustris* BisA53,YP_780232;*Rhodopseudomonas palustris*

BisB5,YP_570899;*Rhodopseudomonas palustris* BisB18,YP_531126;*Rhodopseudomonas palustris*

CGA009,NP_946849;*Rhodopseudomonas palustris* HaA2,YP_487627;*Rhodospirillum rubrum* ATCC

11170,YP_426025;*Roseobacter denitrificans* OCh 114,YP_684125;*Roseobacter* sp.

CCS2,ZP_01750335;*Synechococcus elongatus* PCC 6301,YP_170905;*Synechocystis* sp. PCC

6803,NP_441896;*Synechococcus* sp. WH 5701,ZP_01086192;*Synechococcus* sp. WH

7805,ZP_01124436;*Synechococcus* sp. RS9916,ZP_01470321;*Synechococcus* sp.

RS9917,ZP_01080614;*Synechococcus* sp. RCC307,YP_001227030;*Synechococcus* sp. WH

7803,YP_001225273;*Synechococcus elongatus* PCC 7942,YP_400376;*Synechococcus* sp.

JA-2-3B'a(2-13),YP_477499;*Synechococcus* sp. JA-3-3Ab,YP_474662;*Thalassiosira*

Pseudonana,jgi|Thaps3|14482;*Thermosynechococcus elongatus* BP-1,NP_682635;*Trichodesmium*

erythraeum IMS101,YP_720646;*Vitis vinifera*,CAN75143. Accession numbers for the panel B are as

follows: *Arabidopsis thaliana*,NP_197367;*Bradyrhizobium* sp.

BTAi1,YP_001238876;*Bradyrhizobium* sp. ORS278,YP_001204555;*Chlamydomonas*

Reinhardtii,jgi|Chlre3|195952|RWI_154979;*Chlorobium chlorochromatii*

CaD3,YP_379364;*Chlorobium ferrooxidans* DSM 13031,ZP_01386445;*Chlorobium*

phaeobacteroides BS1,ZP_00532631;*Chlorobium tepidum* TLS,NP_661954;*Erythrobacter* sp.

NAP1,ZP_01040372;*Fulvimarina pelagi* HTCC2506,ZP_01440842;*Congregibacter litoralis*

KT71 ,ZP_01101241;*marine gamma proteobacterium* HTCC2080,ZP_01625489;*Oryza sativa*

(*japonica* cultivar-group),BAF12029;*Ostreococcus lucimarinus* CCE9901,ABO99599;*Ostreococcus*

tauri,jgi|Ostta4|13820;*Pelodictyon luteolum* DSM 273,YP_374402;*Pelodictyon phaeoclathratiforme*

BU-1,ZP_00590062;*Populus trichocarpa*,jgi|Poptr1_1|820932;*Prosthecochloris aestuarii* DSM 271,ZP_00592241;*Prosthecochloris vibrioformis* DSM 265,YP_001130051;*Psychroflexus torquis* ATCC 700755,ZP_01255915;*Rhodobacter sphaeroides* 2.4.1,YP_354588;*Rhodobacter sphaeroides* ATCC 17025,YP_001170066;*Rhodobacter sphaeroides* ATCC 17029,YP_001045650;*Roseovarius* sp. 217,ZP_01037425;*Synechococcus* sp. WH 8102,NP_897056;*Synechococcus* sp. CC9311,YP_730226;*Synechococcus* sp. CC9605,YP_381917;*Synechococcus* sp. CC9902,YP_377382;*Synechococcus* sp. BL107,ZP_01469748;*Thalassiosira pseudonana*,jgi|Thaps3|268474;*Phaeodactylum tricornutum*, jgi|Phatr2|40682;*Vitis vinifera*,CAN63393

Table 1 Distribution of *Slr1923* and *DVR* homologues

+, DVR activity is demonstrated by biochemical or genetical experiments; $\pm$, High sequence similarity to *DVR* or *Slr1923*; - No homologues to *DVR* or *Slr1923*.

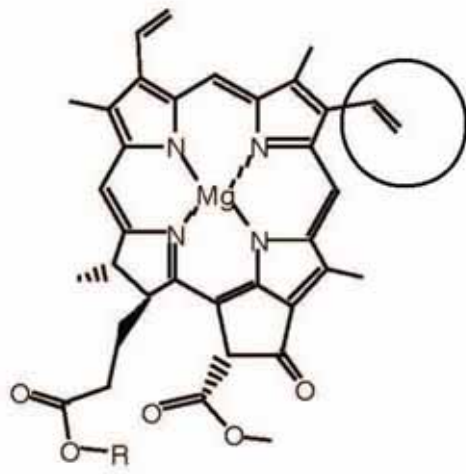
Organisms	Possible Orthologues	
	<i>DVR</i>	<i>Slr1923</i>
Cyanobacteria		
<i>Gloeobacter violaceus</i> PCC 7421	-	+
<i>Anabaena variabilis</i> ATCC 29413	-	+
<i>Synechocystis</i> sp. PCC 6803	-	+
<i>Synechococcus</i> sp. CC 9605	+	-
<i>Synechococcus</i> sp. WH 8102	+	-
<i>Prochlorococcus marinus</i> str. MIT 9312	-	-
<i>Prochlorococcus marinus</i> str. MATL 2A	-	-
Purple bacteria		
<i>Rhodobacter sphaeroides</i> 2.4.1	$\pm$	-
<i>Rhodobacter sphaeroides</i> ATCC 17029	$\pm$	-
<i>Rhodopseudomonas palustris</i> BisA53	-	$\pm$
<i>Rhodopseudomonas palustris</i> BisB5	-	$\pm$
Green filamentous bacteria		
<i>Roseiflexus</i> sp. RS-1	-	-
<i>Chloroflexus aurantiacus</i> J-10-f	-	$\pm$
Green bacteria		
<i>Chlorobium tepidum</i> TLS	+	-
<i>Chlorobium phaeobacteroides</i> DSM 266	-	$\pm$
<i>Chlorobium ferrooxidans</i> DSM 13031	+	$\pm$
<i>Chlorobium chlorochromatii</i> CaD3	+	-

Table 1 Distribution of *Slr1923* and *DVR* homologues

+, DVR activity is demonstrated by biochemical or genetical experiments; $\pm$, High sequence similarity to *DVR* or *Slr1923*; - No homologues to *DVR* or *Slr1923*.

Organisms	Possible Orthologues	
	<i>DVR</i>	<i>Slr1923</i>
Cyanobacteria		
<i>Gloeobacter violaceus</i> PCC 7421	-	+
<i>Anabaena variabilis</i> ATCC 29413	-	+
<i>Synechocystis</i> sp. PCC 6803	-	+
<i>Synechococcus</i> sp. CC 9605	+	-
<i>Synechococcus</i> sp. WH 8102	+	-
<i>Prochlorococcus marinus</i> str. MIT 9312	-	-
<i>Prochlorococcus marinus</i> str. MATL 2A	-	-
Purple bacteria		
<i>Rhodobacter sphaeroides</i> 2.4.1	$\pm$	-
<i>Rhodobacter sphaeroides</i> ATCC 17029	$\pm$	-
<i>Rhodopseudomonas palustris</i> BisA53	-	$\pm$
<i>Rhodopseudomonas palustris</i> BisB5	-	$\pm$
Green filamentous bacteria		
<i>Roseiflexus</i> sp. RS-1	-	-
<i>Chloroflexus aurantiacus</i> J-10-f	-	$\pm$
Green bacteria		
<i>Chlorobium tepidum</i> TLS	+	-
<i>Chlorobium phaeobacteroides</i> DSM 266	-	$\pm$
<i>Chlorobium ferrooxidans</i> DSM 13031	+	$\pm$
<i>Chlorobium chlorochromatii</i> CaD3	+	-

Fig. 1



3,8-divinyl chlorophyll *a*

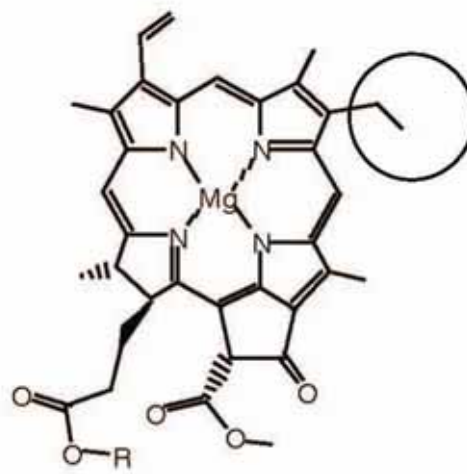
monovinyl chlorophyll *a*

Fig. 2

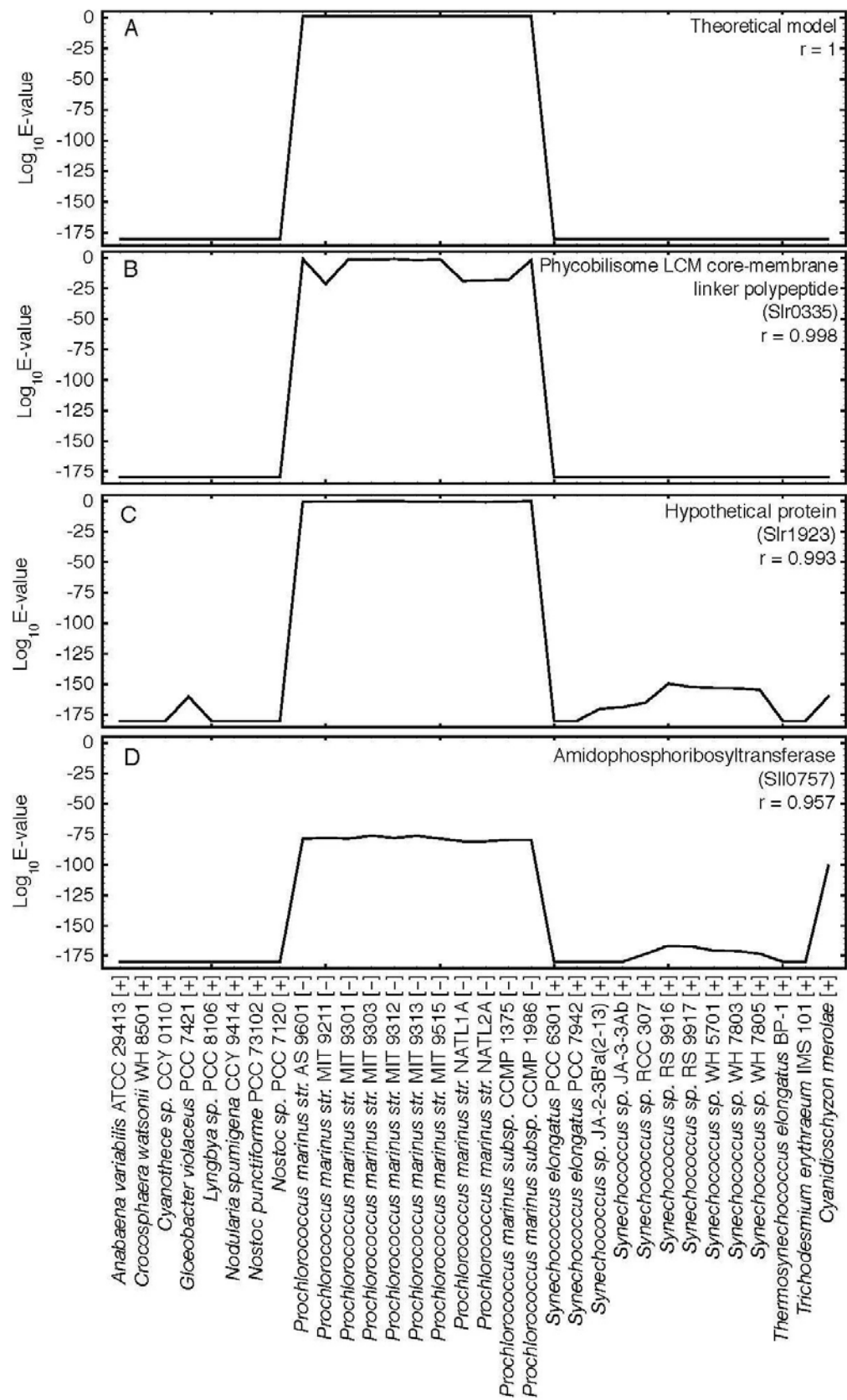


Fig. 3

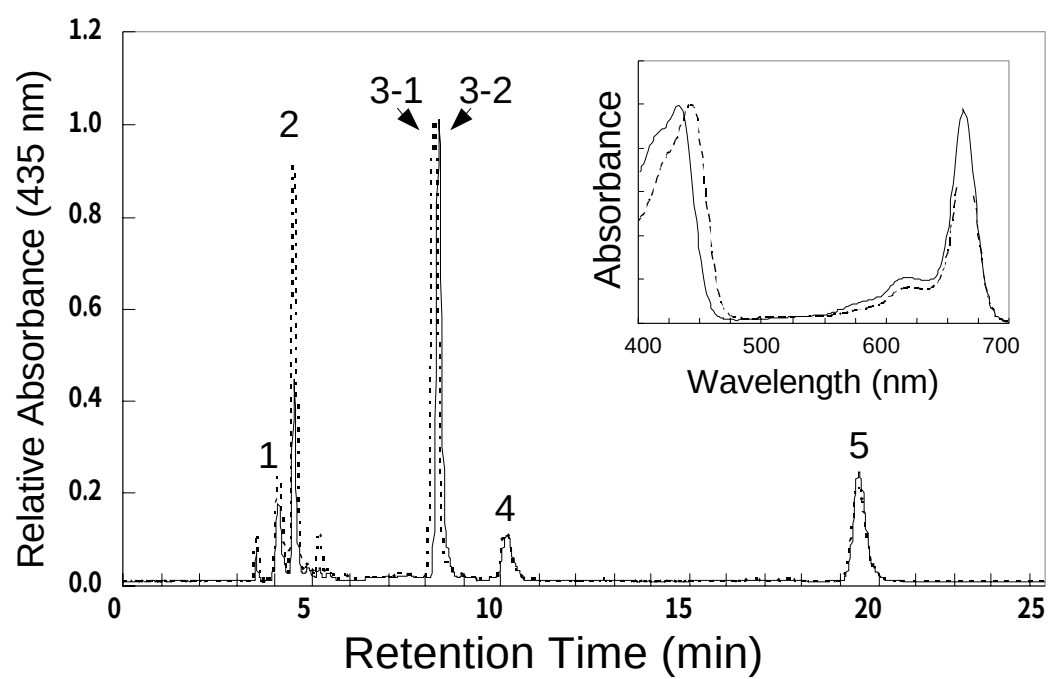


Fig. 4

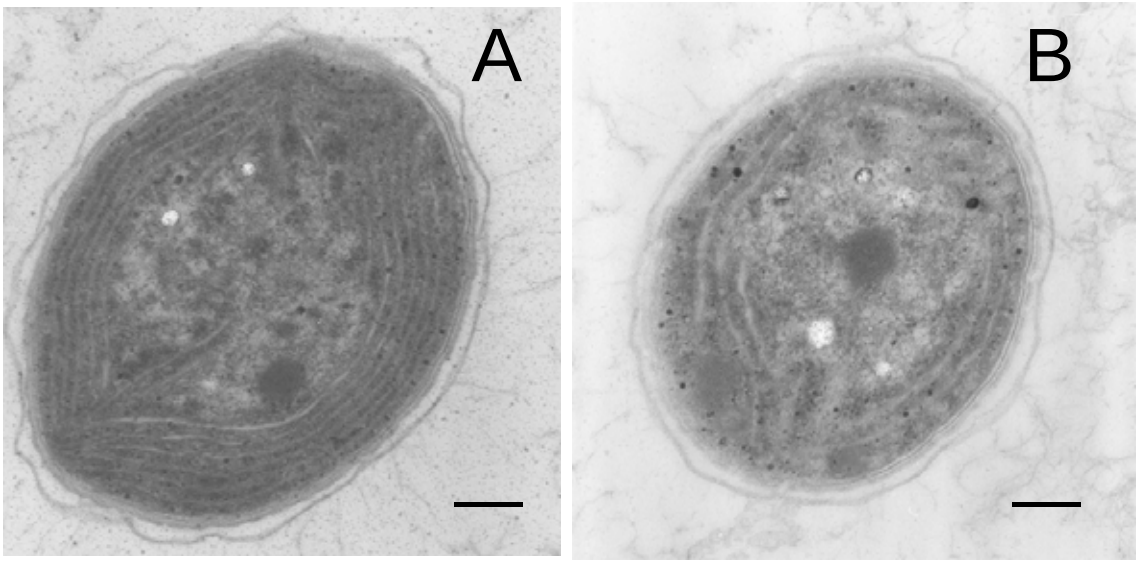


Fig. 5

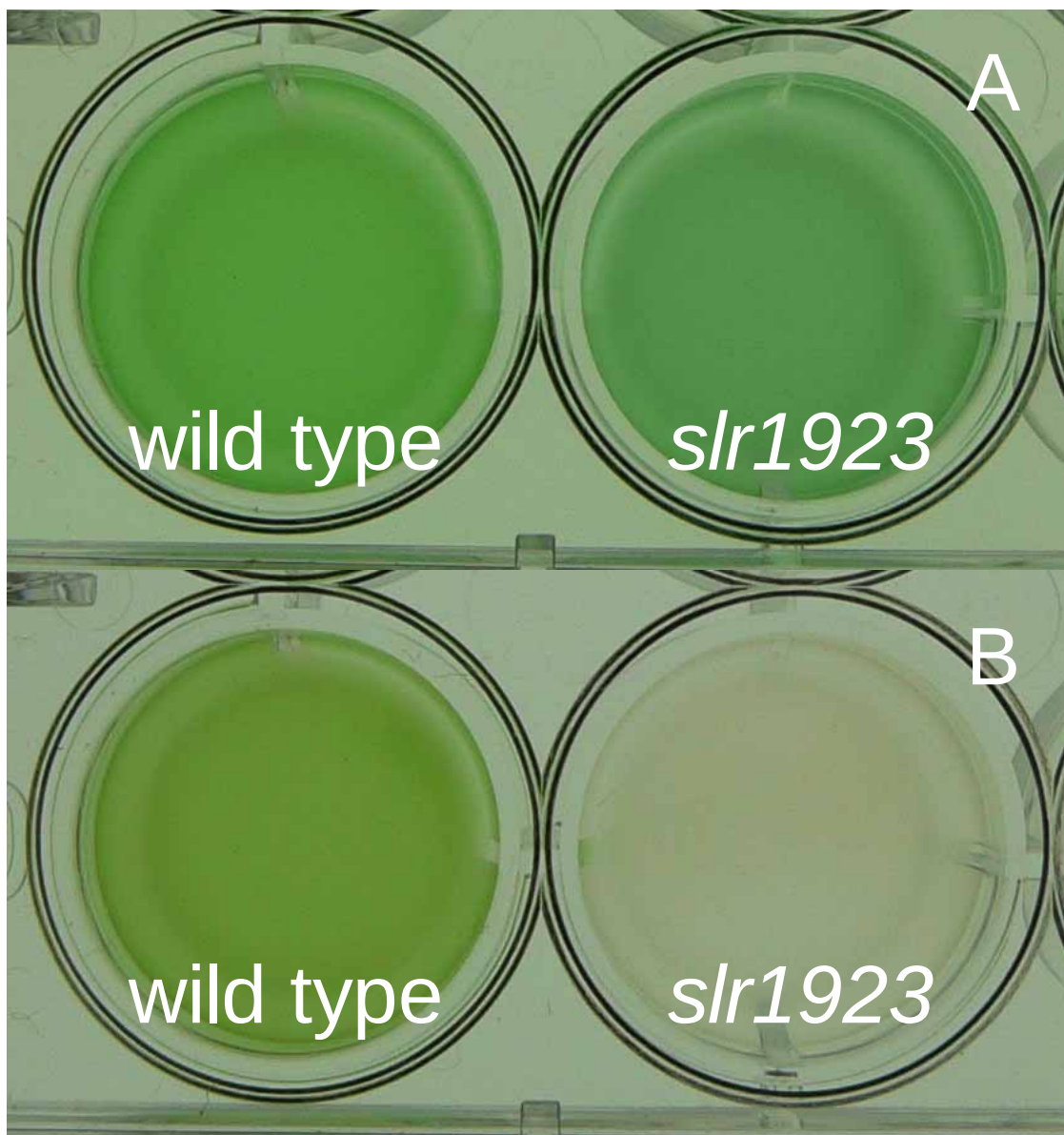


Fig. 6

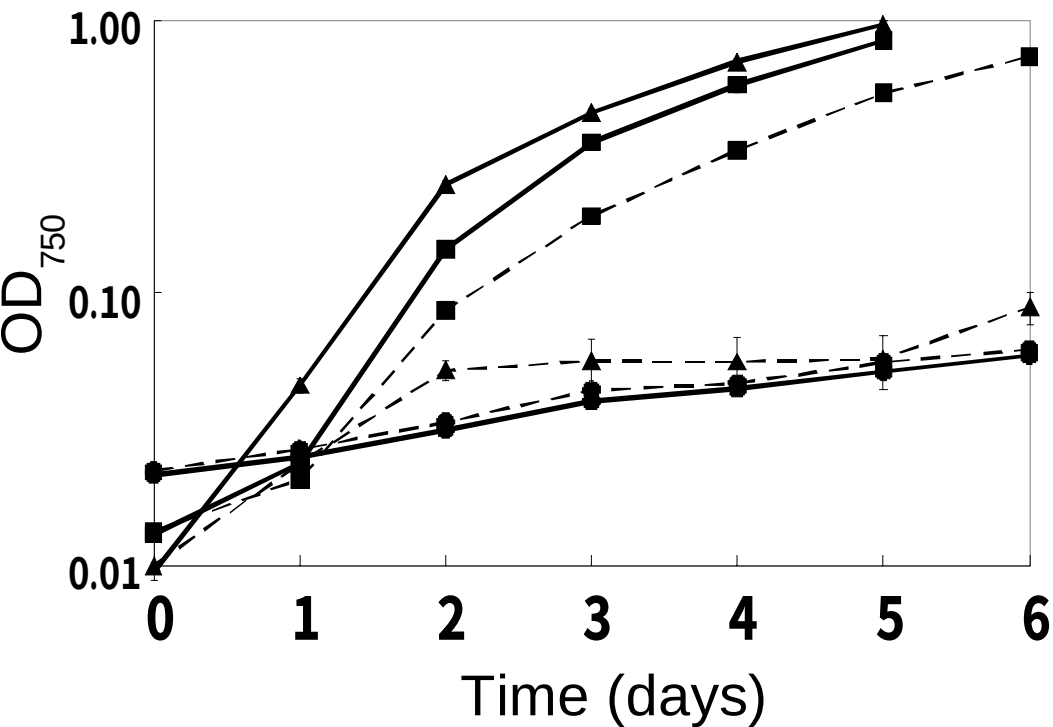


Fig. 7

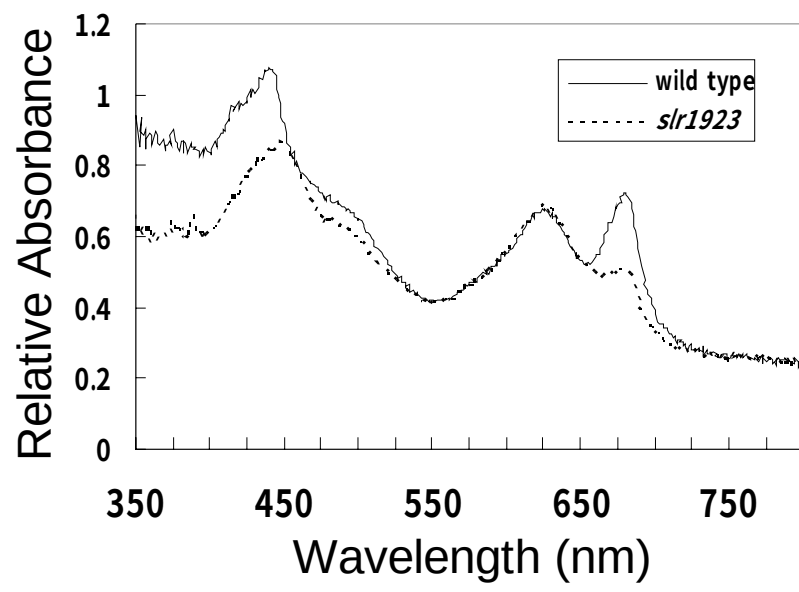


Fig. 8A

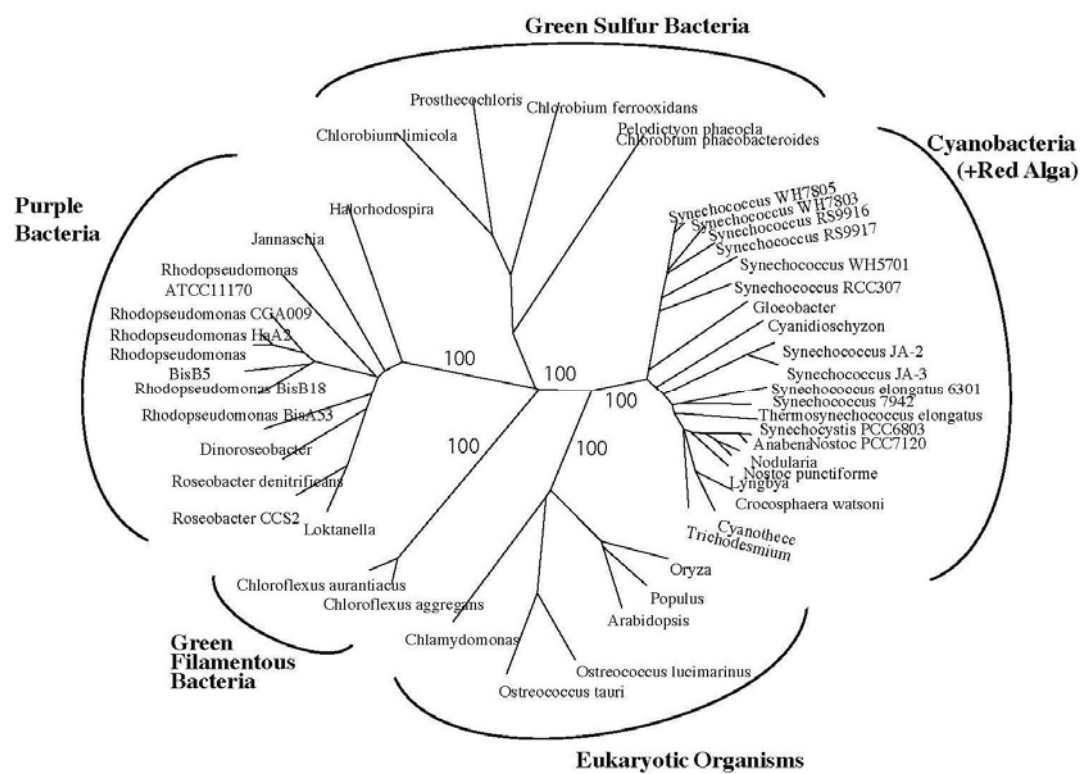
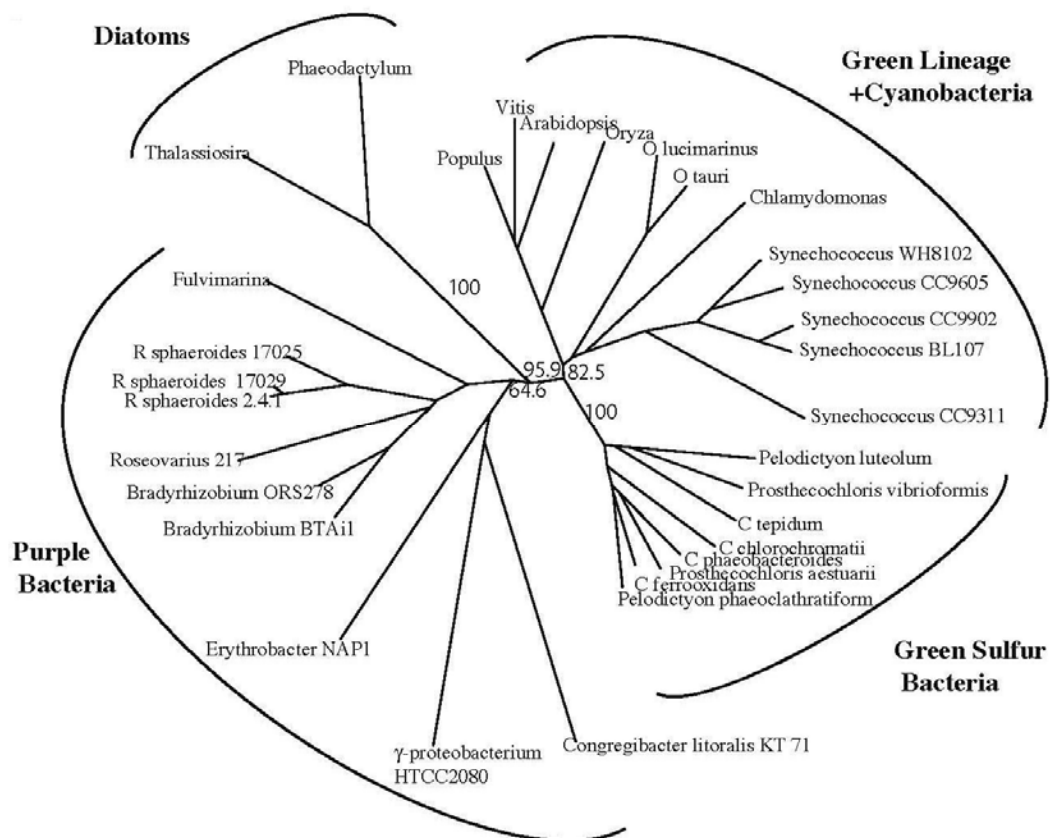
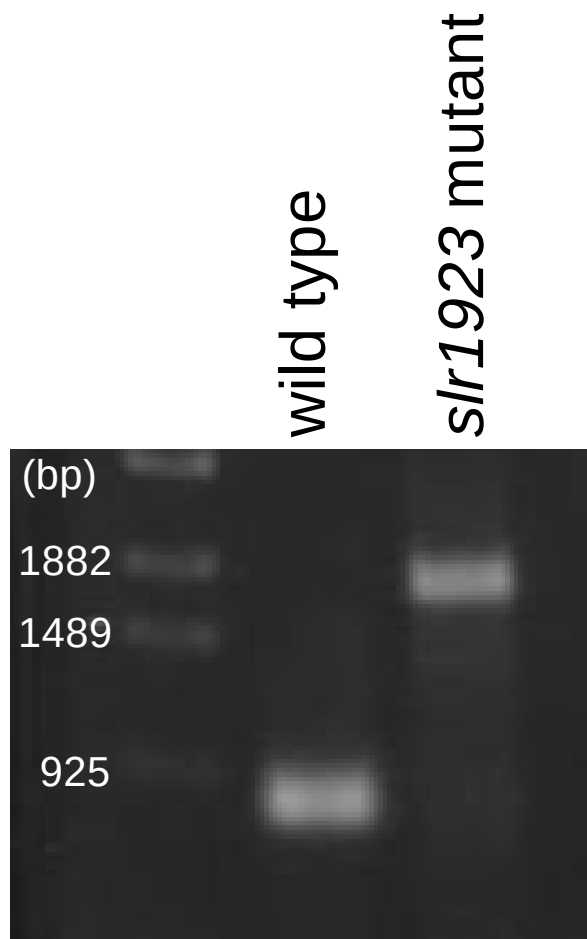


Fig. 8B



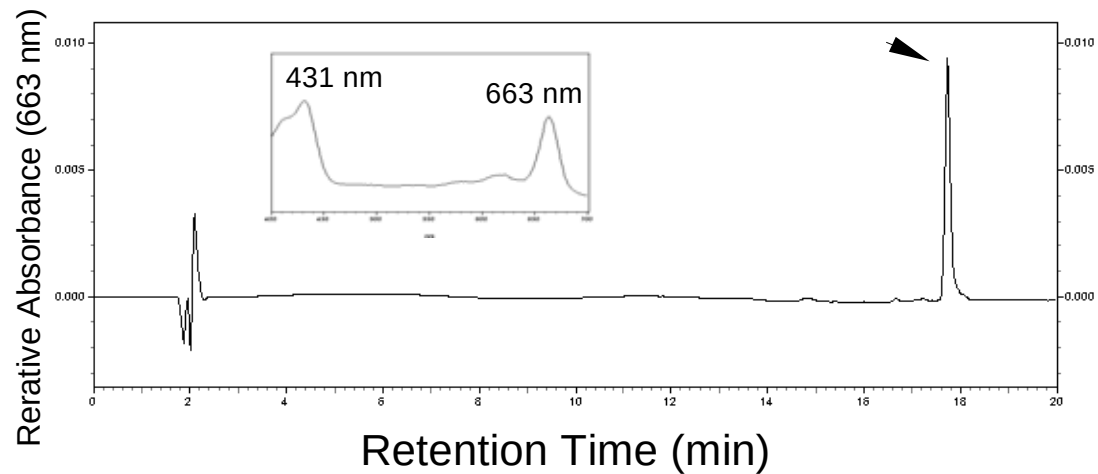
Supplementary Fig. 1

Disruption of the *Slr1923* gene by the insertion of the kanamycin-resistant cassette. To confirm the gene replacement, genomic *Slr1923* fragments were generated by PCR amplification from wild type and *slr1923* mutant colonies. Primers were designed to produce an 800-bp fragment from the wild-type *Slr1923* gene and a 1760-bp fragment from the mutant *slr1923* gene.



Supplementary Fig. 2

Absence of 3,8-divinyl chlorophyll *a* in the *slr1924* knock-out mutant. Pigments were analyzed by HPLC according to the method described in our previous report by Nagata et al (10). The inset shows the absorption spectrum of the chromatographic peak indicated by an arrow. The peak was identified as monovinyl chlorophyll *a* by its retention time and absorption spectrum.



Supplementary Fig. 4

HPLC profiles for the pigments extracted from wild type, the *slr1923*, *sll1224* and *sll1226* knock-out mutants. Pigments were analyzed by HPLC (10). Wild-type, the *sll1224*, and *sll1226* mutant cells accumulated monovinyl chlorophyll *a*, while the *slr1923* mutant cells accumulated 3,8-divinyl chlorophyll *a*.

