slr1923 of *Synechocystis* sp. PCC6803 Is Essential for Conversion of 3,8-Divinyl(proto)chlorophyll(ide) to 3-Monovinyl(proto)chlorophyll(ide)¹

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The deduced amino acid sequence of an slr1923 gene of *Synechocystis* sp. PCC6803 is homologous to archaean F_{420}H_{2} dehydrogenase, which acts as a soluble subcomplex of reduced nicotinamide adenine dinucleotide dehydrogenase complex I. In this study, the gene was inactivated and characteristics of the mutant were analyzed. The mutant grew slower than the wild type under 100 μE m⁻² s⁻¹ but did not grow under high light intensity (300 μE m⁻² s⁻¹). The cellular content of chlorophyll was lower in the mutant, and the absorption spectrum showed a shift in the absorption peak of the Soret band to a longer wavelength by about 10 nm compared with the wild type. It was found, by high-performance liquid chromatography analysis, that the retention time of chlorophyll of the mutant is shorter than that of the wild type and that the peak wavelength of the Soret band was also shifted to a longer wavelength by 11 nm. Proton nuclear magnetic resonance analysis of the chlorophyll of the mutant revealed that the ethyl group of position 8 of ring B is replaced with a vinyl group. The spectrum indicates that the chlorophyll of the mutant is not a normal (3-vinyl)chlorophyll *a* but a 3,8-divinylchlorophyll *a*. These results strongly suggest that the Slr1923 protein is essential for the conversion from divinylchlorophyll(ide) to normal chlorophyll(ide). We thus designate this gene *cvrA* (a gene indispensable for cyanobacterial vinyl reductase).

Land plants, algae, cyanobacteria, and photosynthetic bacteria use various types of chlorophyll molecules (chlorophyll *a*, *b*, *c*, and *d* and bacteriochlorophyll *a*, *b*, *c*, etc.) as inevitable photon-capturing pigments in photosynthesis. Extensive studies with various organisms by biochemical and genetic approaches have disclosed many aspects related to chlorophyll metabolism (for review, see Beale, 1993, 1999; Seng and Smith, 1995). However, the functional or regulatory genes related to various chlorophyll biosynthetic pathways have not yet been fully elucidated. In land plants, chlorophyll precursors are sometimes accumulated as both 3-monovinyl (MV-) or 3,8-divinyl (DV-) intermediates, and the ratio between the two forms changes depending on the species, tissues, and growth conditions (Kim and Rebeiz, 1996). Chlorophyll biosynthetic heterogeneity is assumed to originate mainly in parallel DV- and MV-chl biosynthetic routes interconnected by 8-vinyl reductases (8 VRs) that convert DV-tetrapyrroles to MV-tetrapyrroles by conversion of the vinyl group at position 8 of ring B to the ethyl group. So far, five 8-VR activities have been detected at the levels of DV-Mg-protoporphyrin IX, Mg-protonomonomethyl ester, protochlorophyllide (Pchlide) *a*, chlorophyllide (chlide) *a*, and chl *a* (Kolossov et al., 2006). It has not been clear whether the various 8-VR activities are catalyzed by a single enzyme with broad specificities encoded by a single gene or by a family of enzymes with narrow specificity encoded by multiple genes, as is the case of NADPH Pchlide oxidoreductases (Rebeiz et al., 2003).

Mutants that accumulate DV-chl(ide) *a* can be a potential model system. A mutant that accumulates DV-chl was reported by Bazzaz and Govindjee (1974) and analyzed by Bazzaz (1981) in *Zea mays*. Nagata et al. (2005) and Nakanishi et al. (2005) independently identified gene *AT5G18660* of Arabidopsis as a divinyl reductase (DVR) that has sequence similarity to isoflavone reductase. By investigation of the mutant created by insertional inactivation, Chew and Bryant (2007) demonstrated that *CT1063 (bciA)*, which is an ortholog of the Arabidopsis gene, encodes a C-8 DVR of *Chlorobium tepidum* TLS. They also concluded that BchJ, which had been reported to be a vinyl reductase (Suzuki and Bauer, 1995), is not the enzyme. They assumed that it may play an important role in sub-

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strate channeling and/or the regulation of bacteriochlorophyll biosynthesis.

An ortholog of the DVR gene, however, is not found in the complete genome sequences of cyanobacteria, *Synechocystis sp. PCC6803* (Kaneko et al., 1996), *Anabaena sp. PCC7120* (Kaneko et al., 2001), *Thermosynechococcus elongatus* BP-1 (Nakamura et al., 2002), and *Gloeobacter violaceus PCC7421* (Nakamura et al., 2003), or the unicellular red alga *Cyanidiochyzan merolae* 10D (Matsuzaki et al., 2004). Based on the fact that these organisms synthesize MV-chl(ide), it is reasonable to assume that there exists an 8-vinyl reductase gene(s) whose amino acid sequence is rather different from those found in higher plants or *C. tepidum*. Thus, the identification of other cyanobacterial DVRs is an important subject for elucidation of the chlorophyll biosynthetic pathway.

The model photosynthetic organism *Synechocystis* 6803 has a unique gene, *slr1923*, which encodes a hypothetical protein with unknown function. The deduced amino acid sequence of the gene has a similarity with FpoF, which functions as a F420H2 dehydrogenase subunit F. The protein acts as an electron input device to incomplete NADH dehydrogenase complex 1 (NDH1) of the methanogenic archaeon species *Methanosarcina mazei* (Prommeenate et al., 2004). It is an open question whether it still oxidizes coenzyme F420H2 or evolved differently to use NAD(P)H in *Synechocystis* 6803. Elucidation of a possible function and origin of the protein is of great interest not only with respect to its electron transfer mechanism but also with respect to the evolution and distribution of proteins of archaean origin.

In this study, we created a mutant of *slr1923* of *Synechocystis* 6803 by insertional inactivation, and the characteristics of the mutant were analyzed. It was revealed that all of the molecular species of chl *a* of the mutant were DV-chl *a* but not MV-chl *a* (normal chl *a*). The molecular identity was confirmed by absorption spectra, HPLC, and 1H-NMR analysis. These results show that *slr1923* is inevitable for the conversion of 3,8-DV-(proto)chl(ide) *a* to 3-MV-(proto)chl(ide) *a* in *Synechocystis* 6803. We thus designate the gene *cvrA* (a gene indispensable for cyanobacterial vinyl reductase). The distribution and evolution of vinyl reductases are discussed.

Recently, Ito et al. (2008) considered *slr1923* as a candidate of cyanobacterial DVR from bioinformatics analyses. They also knocked out the gene and characterized the mutant. The basic phenotypes were principally the same, but here we present some more detailed properties and discuss the effects of the mutation.

**RESULTS**

**Construction of the Mutant**

In order to elucidate the role of the protein Slr1923, we have constructed an *slr1923*-inactivated mutant of *Synechocystis* 6803. Wild-type cells were transformed by the plasmid pGEM-Nr-Sp-Cf, in which a spectinomycin resistance cassette with a reverse orientation with respect to the direction of *slr*1923 transcription was inserted (Fig. 1A). After transformation by homologous recombination, colonies grown on a spectinomycin-containing BG11 agar medium were picked up and successive transplantations were performed to segregate the mutant lines. Segregation was confirmed by PCR amplification of the corresponding DNA region by genomic DNA as a template. Figure 1B shows that the amplified band of the mutant was totally replaced by the mutated DNA fragment, indicating total segregation in the mutant.

Figure 1. Construction of the *slr1923* inactivation mutant, segregation of the *slr1923* strain, and northern-blot analysis. A, Schematic representation of the *slr1923*-containing region and construction of the mutant. B, PCR analysis of the segregation of the *slr1923* strain. M1, 100-bp DNA ladder (New England Biolabs); M2, 1-kb DNA ladder (New England Biolabs); W/WT, wild type; M/MT, *slr1923*; Spec, spectinomycin resistance cassette (shaded arrow). C, Northern analysis of transcripts of *slr1923*, *slr1924*, and *slr1925*. RNA was isolated as described in “Materials and Methods,” and total RNA (20 μg) was subjected to analysis. The bottom panels show rRNA stained with methylene blue as a loading control. M, Mutant.
Based on the genomic sequence of *Synechocystis* 6803 (Kaneko et al., 1996), two other genes are located downstream of slr1923 (Fig. 1A). slr1924 is only 25 bp apart from slr1923, and slr1924 and slr1925 overlap by 1 bp. It is possible that these three genes are cotranscribed as a single mRNA. Inactivation of slr1923, the most upstream located among the three genes (Fig. 1A), may affect expression of the downstream genes. Figure 1C shows northern analysis of transcripts of the three genes. The signal intensity of the mutant was about half or lower compared with the wild type. However, the signal intensities of the transcripts were not much different between the three genes. This suggests that expression of slr1924 and slr1925 is not inhibited by disruption of slr1923. Although higher molecular mass signals that correspond to the length equivalent of two genes were observed (Fig. 1C, arrow), further analysis is necessary to determine whether the three open reading frames form operons or not.

Ito et al. (2008) addressed this problem by inactivating slr1924 and slr1925. They found no difference in phenotypes in the two mutants. This observation also supports our results that the phenotype of the mutant observed is not due to lowered or null expression of slr1924 and slr1925 but to inactivation of the slr1923 gene.

Growth Patterns under Different Conditions

When wild-type and mutant cells were grown under 100 μE m⁻² s⁻¹ and aerated by 3% CO₂-containing air (normal condition hereafter), they showed similar growth patterns except that the slr1923 inactivation mutant (*slr1923M*) grew at a slower rate (Fig. 2A). The cells grew exponentially up to about 36 h of cultivation, with doubling times of 10.6 and 12.1 h for the wild type and the mutant, respectively, followed by a gradual decrease in the growth rates, finally reaching a stationary phase after about 80 h.

The growth rates of the wild type under 30, 100, and 300 μE m⁻² s⁻¹ were very similar to those reported previously (Ohtsuka et al., 2004). Under moderate light intensities, the mutant cells grew significantly, although the rates were slower compared with those of the wild type (Table I). When *slr1923M* was grown under strong light (300 μE m⁻² s⁻¹), there was almost no considerable growth from the beginning and the cells started to die at about 34 h of inoculation (Fig. 2B), indicating a high sensitivity to strong illumination. High sensitivity to strong illumination is reported in an Arabidopsis (*Arabidopsis thaliana*) mutant (Nagata et al., 2005; Nakanishi et al., 2005) and also in a *Synechocystis* 6803 mutant (Ito et al., 2008). Although the SD is large, *slr1923M* showed a tendency to grow slower than the wild type under low CO₂ (0.03%). The growth rates under high-salt (0.5 M NaCl) plus low-CO₂ conditions were not much different between the wild type and the mutant. The doubling times of wild-type and mutant cells grown under various conditions are summarized in Table I.

### Spectral Characteristics

An absorption spectrum of wild-type cells showed absorption maxima at 678 and 630 nm in the red region by absorption of chl a and phycobilisomes and at 435 nm in the Soret region (Fig. 3A, solid line). However, the absorption spectrum of *slr1923M* showed a rather low absorbance in the chlorophyll red region, while absorption by phycobilisome was practically unchanged at any stage of growth on the basis of cell number (Fig. 3A, broken line). The peak wavelengths of the red band region due to chl a and phycobilisomes were the same as those of wild-type cells. It was found that the absorption peak of the Soret band shifted to a longer wavelength by about 10 nm. The absorption spectrum of the same mutant reported by Ito et al. (2008) showed a shift by 6 nm in the Soret band. Judged from the absorption spectrum, the peak of the mutant seems to be affected by the higher amount of carotenoids. This may account for the difference of peak positions between our data and those of Ito et al. (2008).
Table I. Doubling times (hours) of wild-type and slr1923M cells grown under various conditions

<table>
<thead>
<tr>
<th>CO₂ Concentration (%)</th>
<th>3</th>
<th>0.03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light Intensity (μE m⁻² s⁻¹)</td>
<td>30 100 300 100</td>
<td>100 100</td>
</tr>
<tr>
<td>NaCl (m):</td>
<td>– – – – 0.5</td>
<td>– 0.5</td>
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<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>slr1923M</th>
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<tbody>
<tr>
<td>24.8 ± 1.1</td>
<td>10.0 ± 0.4</td>
<td>8.8 ± 0.2</td>
</tr>
<tr>
<td>8.8 ± 0.2</td>
<td>12.9 ± 2.5</td>
<td>13.3 ± 3.6</td>
</tr>
<tr>
<td>12.9 ± 2.5</td>
<td>16.7 ± 3.3</td>
<td>16.4 ± 1.8</td>
</tr>
<tr>
<td>16.7 ± 3.3</td>
<td>26.6 ± 1.1</td>
<td>26.6 ± 1.1</td>
</tr>
</tbody>
</table>

*No doubling time, as it died very soon after cultivation.

Figure 3B shows changes of absorption spectra of the mutant cells grown under the high-light condition (300 μE m⁻² s⁻¹). The cells show low chlorophyll absorbance relative to phycobilisome at the initial stage (9.5 h) of cultivation (Fig. 3B, line a). After 17 h, the mutant still retained the spectral characteristics of the initial inoculum (Fig. 3B, line b). However, the color of the culture turned to bluish after 24 h. The spectrum showed a decreased amount of chlorophyll, while phycobilisome content did not change so much based on the number of the cells (Fig. 3B, line c). Finally, the cells lost almost all of the chlorophyll and carotenoids, but a significant amount of phycobilisomes remained. This resulted in total blue color after 33.5 h of growth (Fig. 3B, line d). This is rather different from the responses to high-light illumination of wild-type cells. The wild-type cells show increased carotenoids and decreased phycobilisomes, giving rise to yellowish-green color when grown under strong illumination.

Decreased absorption of the chlorophyll red band relative to phycobilisome could result in higher fluorescence from phycobilisomes and reduced energy transfer efficiency from phycobilisomes to PSII core complexes. This possibility was addressed by measuring fluorescence emission spectra at 77 K. When wild-type cells were excited by a broad-band blue light, three emission bands peaking at 650 to 665, 685 to 695, and 725 nm emitted from phycobiliproteins, phycobilisome anchor (I₁₅₁₈)/PSII, and PSI, respectively, were observed (Fig. 4A, solid line). Fluorescence intensity at 725 nm was about two times higher than that at 695 nm. On the other hand, a very high intensity of fluorescence at around 685 nm relative to that at 725 nm was observed in slr1923M. The intensity of fluorescence at 685 to 695 nm was higher than that emitting from PSI (725 nm), reflecting a higher content of phycobilisome in the cells and less efficiency of energy transfer to the PSII core (Fig. 4A, dotted line). The emission peak corresponding to allophycocyanin was shifted to longer wavelengths in the mutant, indicating that the energy trapped by phycobilisome could not be fully transferred to anchor and/or PSII core complexes. These results indicate that the efficiency of energy transfer from phycobilisomes to PSII is decreased in the mutant.

When cells were excited by violet light to excite mainly the Soret band of chl a, small but distinct emissions at 685 and 695 nm were observed in addition to a large emission peaking at 725 nm in wild-type cells (Fig. 4B, solid line). The emission spectrum of the mutant was practically the same as that of wild-type cells, although emission at 685 nm was higher (Fig. 4B, dotted line), suggesting that the relative content of PSII is little increased in the mutant (see Table IV below). These results indicate that the microenvironment of chlorophylls responsible for emitting those bands is not much altered in the mutant.

Pigment Analysis

The mutant showed a characteristic absorption spectrum. The peak wavelength of chl a of the Soret band is shifted by about 10 nm to a longer wavelength (Fig. 3A, broken line). This suggests that components and/or the composition of the pigments are changed in the mutant. Pigments were extracted from wild-type and mutant cells and their compositions were analyzed by HPLC. On the pigment analysis of the mutant, we recognized that the retention time of chl a (39.4 min) was always a little shorter than that of the wild type (39.9 min; Fig. 5A). The absorption spectra of

![Figure 3](image_url)
separated chlorophyll were different between the wild type and the mutant (Fig. 5B). The spectra showed an absorption maximum at 660 nm in both the wild type and the mutant. The peak wavelength of the Soret band was 431 nm in the wild type; on the other hand, it was shifted to a longer wavelength by 11 nm in the mutant. It is also of note that chl $a$ species were totally replaced by the new chl $a$ species in the mutant. The spectral characteristics and the retention time on HPLC of the modified chlorophyll coincided with those reported by Shedbalkar and Rebeiz (1992), Nagata et al. (2005), and Nakanishi et al. (2005). They identified the modified chlorophyll molecule as a 3,8-DV-chl $a$. It was thus suggested that the chlorophyll species of the mutant is DV-chl $a$, not MV-chl $a$ (normal chl $a$).

Table II shows the cellular contents of chlorophyll and carotenoids obtained by HPLC analysis. The cellular content of $\beta$-carotene was reduced by about 30%, while that of myxoxanthophyll was not much different in the mutant compared with the wild type. Zeaxanthin was increased by about 50% in the mutant, suggesting that light intensity under the “normal” culture condition is strong for the mutant (Schäfer et al., 2006). On the other hand, chlorophyll content was decreased by about 45%. Accordingly, the ratios of myxoxanthophyll and zeaxanthin to chlorophyll were increased by about 1.5 and 2.7 times in the mutant compared with the wild type.

The results obtained by HPLC analysis indicate that inactivation of the slr1923 gene will result in the total replacement of the chlorophyll species from MV type to DV type. The mutant cannot accomplish the reduc-
tion of the vinyl group of position 8 of ring B to an ethyl group.

Analysis of Chlorophyll Molecules by \textsuperscript{1}H-NMR

In order to confirm the molecular species and determine the structure, \textsuperscript{1}H-NMR of the chlorophyll purified from the mutant was measured and compared with that of wild-type chlorophyll.

Figure 6, A and B, shows \textsuperscript{1}H-NMR spectra of chlorophyll from the wild type and the mutant, respectively. In the 7.3 to 7.4 ppm region, the spectrum of wild-type chlorophyll shows signals of a doublet of doublets that is normally observed in MV-chl a (Fig. 6A, inset a). Vinyl proton signals are also exhibited at 5.25 and 5.45 ppm (Fig. 6A, inset b). A resonance signal of the ethyl group connected to position 8 of ring B was also observed in the 0.9 to 0.95 ppm region (Fig. 6A, inset c).

An NMR spectrum of the chlorophyll purified from the mutant showed differences from the wild type at the above characteristic signal regions. The signal appearing in the 7.3 to 7.5 ppm region showed two sets of double doublets, instead of a single doublet of doublets (Fig. 6B, inset a), while their coupling constants were similar to each other. A difference was also observed in the 5.2 to 5.3 ppm region (Fig. 6B, inset b). It exhibited two well-separated sets of double doublets. In addition, a signal originating from the ethyl group connected to position 8 of ring B was also observed in the 0.9 to 0.95 ppm region (Fig. 6A, inset c).

Ito et al. (2008) reached a similar conclusion for the same gene using bioinformatics tools. Their mutant, in which slr1923 was inactivated in Synechocystis 6803, also showed total replacement of the chlorophyll molecule from MV-chl a to DV-chl a.

Photosynthetic Activities and Cellular Contents of Photosystems

The photosynthetic activities or reaction center contents were compared between the wild type and the mutant. The CO\textsubscript{2} fixation rates on the basis of MV-chl a for the wild type and DV-chl a for the mutant were not much different. However, in the mutant, whole chain electron transport activity and segmented electron transport of PSII measured by a Clark-type O\textsubscript{2} electrode were reduced (Table III). The reduced activities could be due to the reduced amount of reaction center or the decreased efficiency of energy transfer from phycobilisome or chlorophyll to the reaction centers. In fact, the light saturation curve of CO\textsubscript{2} fixation activity indicated that the efficiency of utilization of light energy is reduced in the mutant, although the activity is the same at the saturating light intensities (data not shown). It should be noted that we observed a strong dark oxygen consumption activity in the presence of ascorbate and 2,6-dichlorophenolindophenol in the mutant for unknown reasons. This resulted in low activity of PSI electron transport in the mutant.

Relative contents of PSI and PSII in slr1923\textsuperscript{3M} were determined and compared with those in the wild type (Table IV). It was found that PSI and PSII contents on the basis of chlorophyll were not very much different (80%-110% of wild-type values). On a cellular basis, however, both PSI and PSII contents were reduced in the mutant by about 40% and 20%, respectively, compared with the wild type. These results suggest that decreased chlorophyll content on the basis of the cell comes from decreased numbers of active reaction centers but that the number of chlorophylls bound to active reaction center complexes is not changed. These results strongly suggest that the amounts of DV-chl a synthesized are reduced and/or that degradation of the chlorophyll molecule is faster in the mutant.

Phylogenetic Analysis of Slr1923 Homologues and Their Distribution

The results obtained in this study strongly suggest that the slr1923 gene product of Synechocystis 6803 is
Figure 6. $^1$H-NMR spectra of the chl a molecule dissolved in acetone-$D_6$. The chl a molecule was extracted and purified from wild-type or slr1923M cells. The purified chlorophyll was dissolved in deuterated acetone as described in “Materials and Methods,” and $^1$H-NMR was measured for the wild type (A) and slr1923M (B).
essential for the conversion of 3,8-DV-chlorophyll(a) to 3-MV-chlorophyll(a). The Slr1923 protein has a molecular mass of 45 kDa, deduced from its gene sequence, and is a soluble protein, as analyzed by the SOSUI program. The homolog of G. violaceus has a molecular mass of 45 kD, deduced from its gene sequence, and is a soluble protein, as analyzed by the SOSUI program.

On the other hand, the identity and similarity among the homologues indicated that Gll0878 had a four-Cys-containing cluster in the N4 iron-sulfur cluster and transfers electrons from NAD to 50th amino acids, except G. violaceus, which is one of the subunits of NADH dehydrogenase of Thermus thermophilus (Sazanov and Nei, 1987) using F420H2 dehydrogenase subunits. The aligned sequences of the homologues were found in their N-terminal flanking side analyzed by TargetP (http://www.cbs.dtu.dk/services/TargetP).

In addition to the oxygenic photosynthetic organisms, the slr1923 homologues are also found in the genomes of the purple bacteria Rhodopseudomonas palustris CGA009 (RPA1501) and Rhodobacter rubrum (Rru_A0937). Multiple sequence alignment from photosynthetic bacteria to higher plants constructed by ClustalW is shown in Figure 7. The aligned sequences indicate that the homologs have a common sequence cluster showing CXXCX[12]C in the 30th to 50th amino acids, except R. palustris. This sequence motif is found in the N4 iron-sulfur cluster of Nqo3 protein, which is one of the subunits of NADH dehydrogenase of Thermus thermophilus (Sazanov and Hinchliffe, 2006). It is possible that Slr1923 bears an iron-sulfur cluster and transfers electrons from NAD (P)H to the substrate. It should be emphasized that R. palustris also has a four-Cys-containing cluster in the corresponding region, although the motif is different from that mentioned above. Thus, the homolog of R. palustris could also possess the same electron transfer activity. In addition, there are two highly conserved regions within the sequence. They are indicated by boxes in Figure 7. However, no motifs were picked up from the consensus sequences from motif databases.

A phylogenetic tree of Slr1923 homologues was constructed by the neighbor-joining method (Saitou and Nei, 1987) using F_{12},H_{2} dehydrogenase as an outgroup. It indicates that the Slr1923 homologues are separated into two large groups: a purple bacterial group and other photosynthetic organisms.

### Table III. CO₂ fixation activity and electron transport activities in wild-type and slr1923M cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>CO₂ Fixation or Electron Transport Activities</th>
<th>Strain</th>
<th>CO₂ Fixation or Electron Transport Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂O → CO₂</td>
<td>H₂O → MV</td>
<td>H₂O → p-BQ</td>
</tr>
<tr>
<td>Wild type</td>
<td>147.2 ± 8.9</td>
<td>151.0 ± 14.0</td>
<td>137.3 ± 5.8</td>
</tr>
<tr>
<td>slr1923M</td>
<td>153.4 ± 22.7</td>
<td>62.2 ± 7.3</td>
<td>123.3 ± 4.1</td>
</tr>
</tbody>
</table>

### Table IV. Contents of PSI and PSII in wild-type and slr1923M cells on the basis of (MV- or DV-) chl a or cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>PSI Chl a/P700</th>
<th>PSII Chl a/PSII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>99 ± 4 (0.052 ± 0.002)</td>
<td>461 ± 40 (0.011 ± 0.001)</td>
</tr>
<tr>
<td>slr1923M</td>
<td>121 ± 11 (0.031 ± 0.003)</td>
<td>419 ± 13 (0.009 ± 0.003)</td>
</tr>
</tbody>
</table>
Oxygenic photosynthetic organisms form a subcluster closely related to each other. It is of note that among them, a cyanobacterial group and green lineages were clearly separated. A red algal homolog, \textit{CMJ076C} of \textit{C. merolae}, was classified into the cyanobacterial group as reported by Ito et al. (2008). Interestingly, a homolog of the diatom \textit{T. pseudonana} was categorized into a group of green lineages, although diatoms originated by second symbiosis of red algae. This is in sharp contrast with other genes, such as 16S rDNA, PsbA, or PsaA proteins, in which eukaryotic groups branched out from the cyanobacterial group. This suggests that \textit{slr1923} homologues of eukaryotic algae did not originate from some cyanobacterial strain. It is also noted that one of the homologues present in \textit{G. violaceus}, \textit{gll0878}, is classified into the cyanobacterial group, while \textit{glr2543} has highest affinity with the green bacterium \textit{Chlorobium phaeobacteroides} homolog.

Recently, Nagata et al. (2005) and Nakanimi et al. (2005) independently identified DVR in the land plant Arabidopsis. Chew and Bryant (2007) confirmed the presence of the corresponding enzyme in a green sulfur bacterium, \textit{C. tepidum} TL3 (CT1063). On the other hand, Nagata et al. (2005) could not identify DVR homologues in most cyanobacteria. Only a small number of strains, such as \textit{Synechococcus} WH8102 and \textit{Synechococcus} spp. CC9605, CC9902, and CC9311, have DVR homologues. \textit{Slr1923} and its homologues found in other cyanobacteria could be important factors of the second type of vinyl reductase they predicted. Recently, Ito et al. (2008) identified the same gene by means of bioinformatics analysis, which is totally different from our approach.

Interestingly, an \textit{slr1923} homolog was found in the green sulfur bacterium \textit{C. phaeobacteroides} DSM266 (Cpha266_0188). This indicated that the \textit{slr1923} homolog is widely distributed among photosynthetic organisms irrespective of oxygen evolution. Neither the \textit{Slr1923} nor DVR homologous gene is found in divinylchlorophyll-harboring cyanobacteria, \textit{Prochlorococcus}.

DISCUSSION

We have created the \textit{slr1923} inactivation mutant and analyzed its phenotypes. \textit{slr1923} is located within a cluster comprising \textit{slr1924} and \textit{slr1925}, separated by 1000 base pairs. The \textit{slr1924} gene is located 173 bp upstream of \textit{slr1923} (Fig. 8). The \textit{slr1924} gene encodes a 56-kDa polypeptide that has homology to the chloroplast-encoded protein containing a predicted thiol group at a Cys residue, which is conserved in the proteins of the subcluster comprising \textit{slr1924} and \textit{slr1925}, separated by 1000 base pairs. The \textit{slr1924} gene is located 173 bp upstream of \textit{slr1923} and encodes a 56-kDa polypeptide that has homology to the chloroplast-encoded protein containing a predicted thiol group at a Cys residue, which is conserved in the proteins of the subcluster comprising \textit{slr1924} and \textit{slr1925}, separated by 1000 base pairs.
slr1923 Is Essential for Divinylchlorophyll(ide) Reduction


mutant cells, the inactivation of other genes located downstream. The created linker polypeptide, LCM (Fig. 4A). This reflects the incomplete matching of DV-chl a to the binding site will bring the reduced efficiency of energy transfer from phycobilisomes to chlorophyll-protein complexes and the requirement of higher light intensity for the saturation of CO₂ fixation (data not shown). It is also possible that the redox potential of DV-chl a is different from that of MV-chl a and, thus, that the efficiency of the photochemical reaction might be reduced. Determination of the redox potential of the DV-chl a molecule and reaction center is now in progress.

The reduced contents of PSI and PSII on a cell basis (Table IV) can also be accounted for by the replacement of MV-chl a (normal chl a) with DV-chl a. Insufficient spatial fitting of DV-chl a to the binding site will bring about instability of the chlorophyll-protein complexes. Due to their instability, the degradation rate of the complex would be higher than in the wild type. When the mutant cells are grown under strong illumination, the degradation rate becomes further increased due to instability of the chlorophyll-protein complexes. This was found to be the case. The mutant shows a high fluorescence peak at 663 nm emitting from phycobiliproteins at room temperature (data not shown) and at 660 and 685 nm at 77 K emitting from phycobilisomes and the core linker polypeptide, L_cm (Fig. 4A). This reflects the reduced efficiency of energy transfer from phycobilisomes to chlorophyll-protein complexes and the requirement of higher light intensity for the saturation of CO₂ fixation (data not shown). It is also possible that the redox potential of DV-chl a is different from that of MV-chl a and, thus, that the efficiency of the photochemical reaction might be reduced. Determination of the redox potential of the DV-chl a molecule and reaction center is now in progress.

The reduced contents of PSI and PSII on a cell basis (Table IV) can also be accounted for by the replacement of MV-chl a (normal chl a) with DV-chl a. Insufficient spatial fitting of DV-chl a to the binding site will bring about instability of the chlorophyll-protein complexes. Due to their instability, the degradation rate of the complex would be higher than in the wild type. When the mutant cells are grown under strong illumination, the degradation rate becomes further increased due to photodamage of the complex and finally exceeds the complex. Thus, we designate this gene cvrA (a gene indispensable for cyanobacterial vinyl reductase).

The cellular content of DV-chl a of the slr1923M cells was reduced by about half compared with the content of MV-chl a of the wild type. Electron microscopic analysis has shown that the number of thylakoid membranes was reduced in the mutant (Ito et al., 2008). Reduction of chlorophyll content to about half accounts for the reduced number of thylakoid membranes.

It is of note that DV-chl a is incorporated into PSI and PSII complexes and that they seem to be somehow functioning. However, the complexes do not seem to be fully functional and/or they are less stable. The inactivated mutant was sensitive to high-light illumination and started to die under such conditions (Fig. 2B). A similar phenotype was also observed with the dvr (AT5G18660) mutant of Arabidopsis (Nagata et al., 2005) and the slr1923 inactivation mutant of Synechocystis 6803 (Ito et al., 2008). They correlated the sensitivity with the accumulation of DV-chl and assumed that DV-chl completely substitutes for MV-chl in the preexisting protein pigment system and that this substitution leads to photodamage under high-light conditions. The same situation could take place in slr1923M. Ito et al. (2008) considered that free DV-chl a existed in the slr1923 mutant of Synechocystis 6803. Judged from fluorescence spectra at room temperature (data not shown) and 77 K (Fig. 4), however, we did not observe signals consistent with emissions from free chlorophyll. Thus, we conclude that all of the chlorophyll present in the mutant is bound to proteins.

only 25 bp from slr1923 (Fig. 1A). Northern analysis indicated that inactivation of slr1923, which is located upstream of the cluster, does not affect the transcription of other genes located downstream. The created mutant cells, slr1923M, exhibited a characteristic absorption spectrum. It has an absorption maximum at 678 nm in the red band, which is the same as that of the wild type. However, the Soret band was shifted to a longer wavelength by about 10 nm (Fig. 3A). Absorption and ¹H-NMR spectral analyses of the extracted and purified chl a (Figs. 5B and 6) revealed that the chlorophyll species of the mutant is DV-chl a, not MV-chl a (normal chl a). It is also found that all of the chlorophyll species present in the mutant were replaced with DV-chl a (Fig. 5A). According to the pathway of chlorophyll biosynthesis (Kim and Rebeiz, 1996), DV-(P)chl(ide) a is a precursor of MV-(P)chl(ide) a. Judged from the accumulation of DV-chl a in the mutant, it is strongly suggested that Slr1923 is essential to convert 3,8-divinyl(proto)chlorophyll(ide) a to 3-vinyl(proto)chlorophyll(ide) a of Synechocystis 6803. It is highly possible that Slr1923 of Synechocystis 6803 is a divinylchlorophyll(ide) reductase or a part of the

Figure 8. Phylogenetic tree of Slr1923 homologues. A neighbor-joining tree was constructed based on the multiple sequence alignment constructed by the ClustalW algorithm. Archaean homologues were used as an outgroup.
F420 to flavin, a flavin-binding site is expected in the c-terminal side of the Slr1923 protein. The deduced amino acid sequence of slr1923 has homology to that of FpoF protein, which functions as an electron input device of archaean NDH1 as an F_{72}H_{2} oxidoreductase (Prommeenate et al., 2004). In archaea, F_{72} acts as an electron and proton carrier such as NAD(P)\(^{+}\) in bacteria or eukarya. F_{72} has a deazaflavin moiety within the molecule, and the structure of deazaflavin is quite similar to that of flavin. Although the structure of the redox center of F_{72} is similar to that of flavin, the manner of redox reaction is similar to that of NAD(P)\(^{+}\); F_{72} accepts two electrons and one proton, like NAD(P)\(^{+}\) when it is reduced. Taking into account the reports that vinyl reductase is specific to NADPH (Kolossov and Rebeiz, 2001; Nagata et al., 2005; Chew and Bryant, 2007; Ito et al., 2008) and the structural similarity of F_{72} to flavin, a flavin-binding site is expected in the Slr1923 protein. The deduced amino acid sequence shows the motif of a 4Fe4S-type iron-sulfur cluster on its N-terminal side (Fig. 7). This sequence motif is quite similar to that of Nqo3 protein of the N4 iron-sulfur cluster of T. thermophilus (Sazanov and Hinchcliffe, 2006). Thus, the following electron transfer pathway within the Slr1923 protein is assumed: electrons are transferred from NADPH to flavin, which is assumed to be bound to the C-terminal side, and then to the 4Fe4S-type iron-sulfur cluster situated on the N-terminal side.

Another type of vinyl reductase (DVR) has been found in the land plants Arabidopsis and rice and in the marine cyanobacterium Synechococcus WH8102 (Nagata et al., 2005) and some other marine cyanobacteria. It is also found in the green sulfur bacterium C. tepidum TLS (Chew and Bryant, 2007). However, it was not found in other cyanobacteria. The existence of a second vinyl reductase that has low or no homology to DVR was predicted by Nagata et al. (2005), and Ito et al. (2008) reached a similar conclusion from slr1923. Indeed, the slr1923 (cva) homolog was found in other cyanobacteria, in the red alga C. merolae (Matsuzaki et al., 2004), in the diatom T. pseudonana (Armbrust et al., 2004), and in the green algae O. lucimarinus (Palenik et al., 2007) and C. reinhardtii. The deduced amino acid sequences of the homologues of Slr1923 have no homology to that of DVR. It is notable that a strain of green sulfur bacterium, C. phaeobacteroides DSM266, possesses a cva ortholog but not DVR, and the opposite is the case for R. sphaeroides 2.4.1 (Table V).

The distribution of the two genes indicated that the cyanobacteria possess either a dvr or cva homolog, except Prochlorococcus. This clade does not have either gene. On the other hand, green lineages harbor both genes in their nuclei. There seems to be no relationship between the distribution of both proteins and taxonomy (Table V). Taking into account these facts, it is very likely that dvr and cva homologous genes were acquired independently, as pointed out by Ito et al. (2008). However, the real origin of the cva gene is still to be clarified, because the gene is assumed to have originated from archaea.

In green lineages, both dvr and cva homologues are present. If both enzymes are functioning in the chloroplast, inactivation of dvr alone will not suffer from incapability of the conversion from DV-chlide to MV-chlide. However, the DVR-inactivated mutant accumulated only DV-chl and no MV-chl (Nagata et al., 2005; Nakanishi et al., 2005). The following explanations could be possible. (1) The activity of the cva homolog is very low or negligible in higher plant chloroplasts. (2) Expression of the two genes is organ or tissue specific. For example, the dvr gene is expressed mainly in mesophyll cells of leaves, while the cva homolog could be expressed mainly in the epidermis of branches or trunks. (3) Expression depends on developmental stage. In rapidly growing plants, both

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene Name (Gene Identifier)</th>
<th>dvr (bcfA)</th>
<th>cvaA</th>
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<td>-</td>
<td>RPA1501</td>
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<td>C. phaeobacteroides DSM266</td>
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<td>-</td>
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<td>-</td>
<td>glt0878, glr2543</td>
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<td>SYNW 0963</td>
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Table V. Distribution of DVR (bcfA) and cva homologues

* P. marinus = Prochlorococcus marinus.
enzymes seem to be functioning, because DVR activity was observed in both membrane and soluble fractions (Rebeiz et al., 2003). However, in mature leaves, DVR is the only reductase that catalyzes the conversion from DV-(P)chlide to MV-(P)chlide. This hypothesis should be tested in young seedlings or by double mutation. (4) The function of the Slr1923 homolog in green lineages has been altered and the protein does not participate in the reduction of the vinyl group of 3,8-DV-chlide. The wild-type Slr1923 cells were transformed by the resulting plasmid GEM-Nf-Cf, which was linearized by EcoRI digestion. The construct containing recombinant plasmids were cut out by double digestion by EcoRI and SphI. The C-fragment, which was purified by gel electrophoresis, was ligated into gel-purified N-fragment containing pGEM-T vector (pGEM-Nf), giving rise to pGEM-Nf-Cf plasmid. A spectinomycin resistance cassette (S\textsuperscript{r})\textsubscript{176} was inserted between N- and C-fragment-containing recombinant plasmid (pGEM-Nf-Cf), which was linearized by EcoRI digestion. The constructed recombinant vector (pGEM-Nf-N\textsuperscript{r}S\textsubscript{r}Cf) was amplified in E. coli and purified for transformation. The wild-type Synechocystis 6803 cells were transformed by the resulting plasmid pGEM-Nf-N\textsuperscript{r}S\textsubscript{r}Cf vector. The transformed cells, which have a disrupted slr1923 gene, were segregated by successive streaks (about five to six times) on BG11 agar plates that contained 20 μg mL\textsuperscript{-1} spectinomycin. The segregation was confirmed by PCR using N-fragment forward (N-5) \textsuperscript{r} and C-fragment reverse (C-3) primers, with genomic DNA prepared from mutant cells as a template.

**Isolation of Total RNA and Northern Hybridization**

Total RNA from wild-type and mutant cells at mid-log phase was extracted according to Hihara et al. (1998) with some modifications. The RNA was separated by agarose gel electrophoresis, blotted onto a nylon membrane, and fixed by UV illumination. Specific probes for slr1923, slr1924, and slr1925 were prepared by PCR using specific primers. The primers used for the slr1923 probe were the same as those used for the segregation check. The probes for slr1924 and slr1925 were obtained by amplification of the coding region by PCR using primers 5′-ATCTCATTTTATGTTCCG-3′ and 5′-GCCAACGT-TGCTGTGTCG-3′ for slr1924 and 5′-ATGATGGGCTAGATCCC-3′ and 5′-TAATGAACTTATCCTCG-3′ for slr1925. The amplified probes were labeled by digoxigenin following the manufacturer’s instructions (Roche). Luminescence from the hybridized probe was detected with a Bio-Image analyzer (Fuji Photo Film).

**Measurements of Absorption and Fluorescence Spectra**

Absorption spectra of the intact cells were measured with a Shimadzu MPS-2000 spectrophotometer (Shimadzu). Fluorescence emission spectra were recorded by a laboratory-constructed setup at 77 K (Yamane et al., 1997). Excitation light from a 100-W halogen lamp was passed through a Corning 4-96 filter to excite chlorophyll, carotenoids, and phycoerythrin or a combination of Corning 4-96 and Toshiba V-42 filters to excite chlorophyll. The fluorescence intensities were normalized to those of PSI maxima (725 nm).

**Materials and Methods**

**Experimental Organism and Growth Conditions**

A Glc-tolerant Synechocystis 6803 strain was used as the wild-type organism. The cells were grown photoautotrophically in a liquid BG11 medium at 30°C. Cultures were grown under white light at a light intensity of 100 μE m\textsuperscript{-2} s\textsuperscript{-1} under 3% CO\textsubscript{2}-containing air, except where indicated otherwise. For measurements of growth rates, cells were grown in liquid BG11 medium and aliquots of the culture solution were sampled at various time points. The cell density was determined by monitoring the A\textsubscript{680}. **Mutant Construction and Segregation**

The nucleotide sequence of slr1923 was obtained from Cyanobase (http://www.kazusa.or.jp/cya/no/cya.html). Two DNA fragments consisting of 589 bp of the N-terminal side (N-fragment, Nf) and 557 bp of the C-terminal side (C-fragment, Cf) of the slr1923 gene were amplified by PCR separately. For N-fragment amplification, forward (N-5) \textsuperscript{r} and reverse (N-3) \textsuperscript{r} primers with the sequences 5′-CATGACCGCTTCGTGCCC-3′ and 5′-GGGGAAATCTCCACAACAGGGGCTGTCCTCC-3′, respectively, and for the C-fragment, forward (C-5) \textsuperscript{r} and reverse (C-3) \textsuperscript{r} primers with the sequences 5′-GGGGAAATCTCAATGTTCCCGGGCTCTGGC-3′ and 5′-GAGGGACGTGGTCAGCC-3′, respectively, were used. The EcoRI recognition site was introduced into the 5′ end of reverse (N-3) \textsuperscript{r} and forward (C-5) \textsuperscript{r} primers for the N- and C-fragments, respectively. The amplified N- and C-fragments were cloned separately into the pGEM-T vector (Promega) and introduced into competent Escherichia coli cells (XL-1 blue). N- and C-fragment-containing recombinant plasmids were cut out by double digestion by EcoRI and SphI. The C-fragment, which was purified by gel electrophoresis, was ligated into gel-purified N-fragment containing pGEM-T vector (pGEM-Nf), giving rise to pGEM-Nf-Cf plasmid.

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**Pigment Analysis**

A cell suspension (1.3 mL) in which the A\textsubscript{680} was adjusted to 0.2 was precipitated, and then 10 μL of pure water and 190 μL of dimethyl formamide were added. After a short vortexing, the solution was transferred to a brown plastic bottle and kept at −20°C overnight. After a short vortexing, an aliquot of 50 μL was withdrawn and centrifuged. Ten microliters of the supernatant was injected onto an HPLC column (Phenomenex). Other chemicals and instrumental settings were as described by Kashiho and Kudoh (2003). Data were transferred to a personal computer, and the amounts of the pigments were calculated from their extinction coefficients at the monitoring wavelength.

**Preparation of Thylakoid Membranes**

Cells were harvested by centrifugation (3,200×g, 5 min) and washed in buffer A (25% [w/v] glycerol, 10 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, and 50 mM MES-NaOH [pH 6.5]). The precipitated cells were resuspended in buffer A. The cells were ruptured by zirconia beads (d = 0.1 mm, 1.6 g mL\textsuperscript{-1} cell suspension) with the Mini Bead Beater (Coiler-Parmer). Agitation was performed for 20 s followed by cooling on ice for 1 min to minimize heat denaturation. This cycle was repeated three times. The supernatant after precipitation of zirconia beads was recovered and centrifuged at 6,000×g for 10 min at 4°C to remove unbroken cells. Thylakoid membranes and soluble fractions were separated by centrifugation at 300,000×g for 30 min. The thylakoid membranes recovered as a precipitate were resuspended in buffer A and stored at −80°C until use.

**Chlorophyll Purification and NMR Analysis**

Chlorophylls were extracted by sonicating the mixture of 1.5 mL of thylakoid membrane preparation and 6.0 mL of acetone-Na\textsubscript{2}HPO\textsubscript{4} for 5 min. The resulting solution was filtered by a filter paper and then a glass filter. Filterate volume was adjusted to 10 mL with acetone, and 1.33 mL of dioxane was added with stirring on ice. Then, water was slowly added until green aggregates were formed. Samples were kept at −20°C for 1 h and centrifuged at 2,300×g for 15 min at 4°C. The green pellet was dissolved in 3 mL of ethanol and again filtered through a glass filter. Chlorophyll purification was repeated to remove contaminating carotenoids. Finally, purified chlorophyll was lyophilized overnight. Dried samples were dissolved in 700 μL of NMR-grade deuterated acetone. Two hundred microliters of the sample was loaded in the NMR machine. 1H-NMR was measured on a JEOL ECP-600 at 600 MHz using a solvent peak as a reference (J = 2.00).

**Measurement of Oxygen Evolution or Consumption Activities**

Cells were suspended in liquid BG11 medium, and chlorophyll concentration was adjusted to 20 μg mL\textsuperscript{-1}. Oxygen evolution or consumption activities were measured by a Clark-type oxygen electrode (Rank Brothers) as described previously (Inoue et al., 2001).

**Determination of PSI and PSII**

For the determination of PSI content, light-induced absorption changes of P700 were measured at 703 nm by a Shimadzu MPS-2000 spectrophotometer (Shimadzu). Fluorescence emission spectra were recorded by a laboratory-constructed setup at 77 K (Yamane et al., 1997). Excitation light from a 100-W halogen lamp was passed through a Corning 4-96 filter to excite chlorophyll, carotenoids, and phycoerythrin or a combination of Corning 4-96 and Toshiba V-42 filters to excite chlorophyll. The fluorescence intensities were normalized to those of PSI maxima (725 nm).

**Cyanobacterial chlorophyll a/b content was measured using a slightly modified version of the method of Nakayama et al. (1979).**
Phylogenetic Analysis of slr1923 Homologues

The deduced amino acid sequences of slr1923 homologues were trimmed to the predicted mature forms by TargetP (http://www.cbs.dtu.dk/services/TargetP/) for nucleus-encoded genes. The amino acid sequences were aligned by the ClustalW algorithm. A neighbor-joining tree was constructed based on the ClustalW alignment.

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