<table>
<thead>
<tr>
<th>Title</th>
<th>Oxidation of P700 in Photosystem I Is Essential for the Growth of Cyanobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Shimakawa, Ginga / Shaku, Keiichiro / Miyake, Chikahiro</td>
</tr>
<tr>
<td>Citation</td>
<td>Plant Physiology, 172(3): 1443-1450</td>
</tr>
<tr>
<td>Issue date</td>
<td>2016-11</td>
</tr>
<tr>
<td>Resource Type</td>
<td>Journal Article / 学術雑誌論文</td>
</tr>
<tr>
<td>Resource Version</td>
<td>publisher</td>
</tr>
<tr>
<td>Rights</td>
<td>©2016 American Society of Plant Biologists</td>
</tr>
<tr>
<td>DOI</td>
<td>10.1104/pp.16.01227</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://www.lib.kobe-u.ac.jp/handle_kernel/90003823">http://www.lib.kobe-u.ac.jp/handle_kernel/90003823</a></td>
</tr>
</tbody>
</table>

PDF issue: 2018-11-27
Oxidation of P700 in Photosystem I Is Essential for the Growth of Cyanobacteria

Ginga Shimakawa, Keiichiro Shaku, and Chikahiro Miyake*

Department of Biological and Environmental Science, Faculty of Agriculture, Graduate School of Agricultural Science, Kobe University, Nada-ku, Kobe 657–8501, Japan (G.S., K.S., C.M.); and Core Research for Environmental Science and Technology, Japan Science and Technology Agency, Chiyoda-ku, Tokyo 102–0076, Japan (C.M.)

ORCID ID: 0000-0002-2426-2377 (C.M.).

The photoinhibition of photosystem I (PSI) is lethal to oxygenic phototrophs. Nevertheless, it is unclear how photodamage occurs or how oxygenic phototrophs prevent it. Here, we provide evidence that keeping P700 (the reaction center chlorophyll in PSI) oxidized protects PSI. Previous studies have suggested that PSI photoinhibition does not occur in the two model cyanobacteria, Synechocystis sp. PCC 6803 and Synechococcus elongatus PCC 7942, when photosynthetic CO2 fixation was suppressed under low CO2 partial pressure even in mutants deficient in flavodoxin protein (FLV), which mediates alternative electron flow. The lack of FLV in Synechococcus sp. PCC 7002 (S. 7002), however, is linked directly to reduced growth and PSI photodamage under CO2-limiting conditions. Unlike Synechocystis sp. PCC 6803 and S. elongatus PCC 7942, S. 7002 reduced P700 during CO2-limited illumination in the absence of FLV, resulting in decreases in both PSI and photosynthetic activities. Even at normal air CO2 concentration, the growth of S. 7002 mutant was retarded relative to that of the wild type. Therefore, P700 oxidation is essential for protecting PSI against photoinhibition. Here, we present various strategies to alleviate PSI photoinhibition in cyanobacteria.

Low CO2 fixation efficiency in the Calvin-Benson cycle prevents the utilization of NADPH and ATP in photosynthesis and causes these molecules to accumulate, resulting in oxidative photosynthetic cell damage. High light, low temperature, and CO2 limitation increase NADPH and ATP levels beyond the Calvin-Benson cycle requirements. Electrons and H+ accumulate in the photosynthetic electron transport (PET) system. Excess electrons in the PET system trigger oxidative damage to PSI by forming reactive oxygen species (ROS), including the superoxide anion radical (O2•−) and singlet oxygen (1O2), within PSI and degrading the P700 reaction center chlorophyll (P700); Sonoike, 1996; Sejima et al., 2014; Zivcak et al., 2015a, 2015b; Takagi et al., 2016). PSI repair has been reported to be a slow process (Kudoh and Sonoike, 2002), and a recent study showed that it took more than 12 d for damaged PSI in wheat (Triticum aestivum) leaves to recover completely (Zivcak et al., 2015b). PSI photoinhibition, therefore, is very detrimental to oxygenic phototroph growth. Nevertheless, PSI photoinhibition is alleviated by keeping P700 oxidized (Sejima et al., 2014).

In the PET system of oxygenic phototrophs, P700 oxidation is a physiological response to environmental variations. In C3 plants, low CO2 and/or high light intensity induce P700 oxidation in vivo (Klughammer and Schreiber, 1994; Laisk and Oja, 1994; Miyake et al., 2004, 2005). Several molecular mechanisms are proposed for P700 oxidation wherein the PSI acceptor does not limit the PET reaction. First, H+ accumulation on the luminal side of thylakoid membranes lowers reduced plastoquinone (plastoquinol) oxidation rates in the cytochrome (Cyt) b6/f complex (Kramer et al., 1999). Second, plastidial terminal oxidase and cyanobacterial respiratory terminal oxidases on the thylakoid membranes suppress PSI electron influx by accepting upstream PSI electrons in the PET system. Oxygen is the final electron acceptor (Beardall et al., 2003; Trouillard et al., 2012; Lea-Smith et al., 2013). Finally, plastoquinol accumulation inhibits the Q-cycle turnover in the Cyt b6/f complex, which suppresses electron flow from the Cyt b6/f complex to P700. This reaction is called the reduction-induced suppression of electron flow (RISE; Shaku et al., 2016). Overall, these molecular mechanisms contribute to P700 oxidation, thereby preventing PSI photoinhibition and enabling oxygenic phototrophs to thrive. The proton gradient regulation5 (pgr5) mutant of Arabidopsis (Arabidopsis thaliana) cannot
keep P700 oxidized and shows PSI photoinhibition under high-light and fluctuating light conditions (Munekage et al., 2002; Suorsa et al., 2012), which shows the importance of the oxidation of P700 for the protection of PSI in plants.

Unlike green plants, P700 oxidation mechanisms in cyanobacteria are unclear. It is known that flavodiiron protein (FLV) could contribute to P700 oxidation. Four FLV isoforms (FLV1−FLV4) have been identified in the model cyanobacterium *Synechocystis* sp. PCC 6803 (S. 6803; Helman et al., 2003). FLV1 and FLV3 (FLV1/3) function as a heterodimer and catalyze the reduction of NAD(P)H as electron donors (Vicente et al., 2002; Helman 2003). FLV-dependent electron fluxes are coupled to photosynthesis and should alleviate electron overaccumulation in PSI (Munekage et al., 2002; Suorsa et al., 2012), which demonstrates that the deletion of FLV in S. 7002 rendered it unable to oxidize P700, resulting in PSI photoinhibition. These findings show that there are different strategies in cyanobacteria to protect PSI against photooxidative damage under CO2 limitation.

## RESULTS

### Effects of FLV on the Growth of S. 7002 under Ambient [CO2]

We constructed the S. 7002 mutant, Δflv1/3, which lacks the *flv1* and *flv3* orthologs present in S. 6803 (SYNPC7002_A1743 and SYNPC7002_A1321; Supplemental Fig. S1). We found that the growth of S. 7002 Δflv1/3 is slower than that of the wild type under ambient [CO2] (Fig. 1). This response was not observed in either S. 6803 (Zhang et al., 2009) or S. 7942 (Shaku et al., 2015). Approximately 2 weeks after inoculation, the OD750 for Δflv1/3 was 70% lower than that for the wild type (Fig. 1A). The chlorophyll (Chl) content in the Δflv1/3 medium was half that of the wild type (Fig. 1B). These results indicate that S. 7002 requires FLV1/3 for optimal growth under ambient [CO2].

### Effects of CO2 Limitation on Photosynthetic Parameters in Wild-Type and Δflv1/3 S. 7002

We hypothesized that PSI photoinhibition occurs under CO2 limitation in the absence of FLV-mediated AEF in S. 7002. We studied the effects of CO2 limitation on total oxidizable P700 and net photosynthetic oxygen evolution rates in S. 7002 wild type and Δflv1/3. After a 2-h exposure to CO2 limitation, neither PSI nor photosynthesis inactivation was detected in S. 7002 wild type (Fig. 2, A and B). Nevertheless, a significant posttreatment reduction in total oxidizable P700 (Fig. 2A) and suppression of photosynthesis (Fig. 2C) were observed in S. 7002 Δflv1/3. The dramatic decreases in photosynthetic parameters (0%–10% of pretreatment levels; Fig. 2, A and C) for Δflv1/3 indicate that the lack of FLV1/3 in S. 7002 causes severe PSI photooxidation under CO2 limitation.

To determine whether the deletion of FLV-mediated AEF combined with CO2 limitation always causes PSI photoinhibition in cyanobacteria, we applied the same treatment to S. 6803 and S. 7942. For S. 6803, we used a

<table>
<thead>
<tr>
<th>Cyanobacteria</th>
<th>Flavodiiron Proteins</th>
<th>Respiratory Terminal Oxidases</th>
<th>Cyclic Electron Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>flv1/3</em></td>
<td><em>flv2/4</em></td>
<td><em>cox</em></td>
</tr>
<tr>
<td>S. 7002</td>
<td>A1743/A1321</td>
<td>A1162 − A1164</td>
<td>−</td>
</tr>
<tr>
<td>(SYNPC7002_</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. 6803</td>
<td>sll1521/sll0550</td>
<td>sll0219/sll0217</td>
<td>slr1136 − slr1138</td>
</tr>
<tr>
<td>S. 7942</td>
<td>1810/1809</td>
<td>−</td>
<td>2602 − 2604</td>
</tr>
</tbody>
</table>
mutant deficient in the expression of all four \textit{flv} genes (\textit{\Delta flv1/3/4}), since the wild type of this species possesses \textit{FLV2/4} (Table I; Supplemental Fig. S1; Eisenhut et al., 2012). Unlike \textit{S. 7002}, the amounts of total oxidizable P700 were the same before and after the treatment for both \textit{S. 6803} and \textit{S. 7942} even when the \textit{flv} genes were not expressed (Supplemental Fig. S2, A and B). CO2 limitation also did not affect the dependence of photosynthetic oxygen evolution rates on photon flux density in either the wild type or the \textit{flv} mutants of \textit{S. 6803} and \textit{S. 7942} (Supplemental Fig. S2, C–F). On the other hand, the deletion of \textit{FLV1/3} in \textit{S. 7942} decreased photosynthetic oxygen evolution rates even before CO2 limitation, particularly in high-light conditions (Supplemental Fig. S2, D and F), due to RISE (Shaku et al., 2015).

Effects of \textit{FLV} on the Photosynthetic Parameters of PSII and PSI in \textit{S. 7002}

To determine the relationship between PSI photodamage and P700 oxidation in \textit{S. 7002}, we simultaneously monitored Chl fluorescence and the P700 redox state in PSI during the transition from CO2 saturation to CO2 limitation. We modified methods used in our previous

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Growth of \textit{S. 7002} wild type and the mutant \textit{\Delta flv1/3} under ambient CO2. Optical density at 750 nm (OD$_{750}$; A) and Chl (B) measurements were independently conducted three times, and the data are shown as means ± ss. Black circles, \textit{S. 7002} wild type; red triangles, \textit{\Delta flv1/3}. Differences between \textit{S. 7002} wild type and \textit{\Delta flv1/3} were analyzed by Student’s \textit{t} test. Asterisks indicate statistically significant differences between \textit{S. 7002} wild type and \textit{\Delta flv1/3} at \textit{P} < 0.05.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Reduced activities of PSI and photosynthesis in \textit{S. 7002} wild type (WT) and \textit{\Delta flv1/3} after 2-h exposures to CO2 limitation during illumination (290 \textit{\mu}mol photons m$^{-2}$ s$^{-1}$). The reaction mixture contained fresh A medium and cyanobacterial cells (10 \textit{\mu}g Chl mL$^{-1}$). Residual total oxidizable P700 (A) and photosynthetic oxygen evolution rates (B and C) were measured before and after 1 h in the dark following treatments. Black and red symbols represent the wild type and \textit{\Delta flv1/3}, respectively. Closed and open symbols represent the data before and after the treatments (B and C), respectively. Photosynthetic oxygen evolution rates were measured in the presence of 10 \text{mM NaHCO}_3. Each measurement was conducted three times, and means ± ss are shown. Differences between the data before and after the treatments were analyzed by Student’s \textit{t} test. Asterisks indicate statistically significant differences at \textit{P} < 0.05.}
\end{figure}
work (Shimakawa et al., 2015). Upon red actinic light (AL) illumination in S. 7002 wild type, incident quantum yields of PSI [Y(I)] and PSII [Y(II)] rose (by about 0.8 and 0.3, respectively). Thereafter, they began to decline (to about 0.6 and 0.1, respectively) due to a decrease in photosynthesis (Fig. 3A). CO₂ consumption suppressed photosynthesis. Y(I) and Y(II) were restored when CO₂ was added in the form of NaHCO₃ [Fig. 3A; Hayashi et al., 2014; Shimakawa et al., 2015]. The P700 redox state also responded to CO₂ limitation. The suppression of photosynthetic linear electron flow increased the yield of oxidized P700 [Y(ND)]. This condition was alleviated by the addition of CO₂ [Fig. 3A]. On the other hand, the yield of photoexcited P700 [Y(NA)] did not change in response to the shortage of CO₂ [Fig. 3A]. Therefore, the PSI acceptor side limitation did not change after S. 7002 wild type was subjected to CO₂ limitation. It is unclear why Y(I) was significantly higher than Y(II) in this study. Cyclic electron flow around PSI may contribute to surplus Y(I) (see “Discussion”). The S. 6803 mutant ΔndhD1/2, which is deficient in the D subunits of NAD(P)H dehydrogenase, however, also had higher Y(I) than Y(II) (Supplemental Fig. S3). The large gap between Y(I) and Y(II) in cyanobacteria merits further investigation.

Next, we measured the photosynthetic parameters of PSII and PSI after the transition to CO₂ limitation in S. 7002 Δflv1/3. Before CO₂ deprivation, Y(I) and Y(II) in Δflv1/3 were lower (about 0.6 and 0.2, respectively) than those in the wild type, whereas Y(NA) was higher in the mutant (about 0.3) than in the wild type (about 0.1; Fig. 3). These results imply that FLV1/3 drives AEF in S. 7002, as it does for S. 6803 (Helman et al., 2003) and S. 7942 (Shaku et al., 2015). CO₂ limitation did not induce P700 oxidation in S. 7002 Δflv1/3 [Fig. 3B]. An increase in Y(NA) indicated that the electron flux from P700 to the acceptor side of PSI was reduced further still (Fig. 3B). Y(I) also was considerably suppressed under CO₂ limitation (Fig. 3B). The addition of NaHCO₃ did not restore Y(I) or Y(II) (Fig. 3B). These results suggest that, unless FLV1/3-mediated AEF is active, PSI photo-inhibition occurs in S. 7002 during CO₂ limitation. For S. 7002, FLV1/3 plays a primary role in oxidizing the PET system under CO₂ limitation. Recently, we found that S. 7002 drives an oxygen-dependent AEF to restore linear electron transport during CO₂-limited photosynthesis. This process is particularly evident in cells grown under ambient [CO₂] (Shimakawa et al., 2016). In this study, a simultaneous measurement of oxygen concentration and Chl fluorescence was performed in S. 7002 Δflv1/3 grown under ambient [CO₂] (Supplemental Fig. S4), indicating that FLV1/3 is the molecular mechanism of the oxygen-dependent AEF we found in S. 7002 (Shimakawa et al., 2016).

Effects of FLV on the Photosynthetic Parameters of PSII and PSI in S. 6803 and S. 7942

The photosynthetic parameters of PSII and PSI responded differently to CO₂ limitation in S. 6803 than they did in S. 7002. In S. 6803 wild type, Y(I) and Y(II) decreased to minimum values and then started to recover, reaching approximately 90% and 60% of the initial values, respectively, without the addition of NaHCO₃ (Supplemental Fig. S5A). This recovery occurred due to the activation of an oxygen-dependent AEF driven by FLV2/4 but not by FLV1/3 (Shimakawa et al., 2015; Supplemental Figs. S5 and S6). The AEF stimulated linear electron flow, which decreased both Y(ND) and Y(NA) (Supplemental Fig. S5A). In S. 6803, the deletion of FLV1/3 reduced both Y(I) and Y(II) relative to the wild type before CO₂ consumption (Supplemental Fig. S5B). For S. 6803 Δflv1/3 before CO₂ depletion, Y(ND) was lower than that of the S. 6803 wild type, whereas Y(NA) in the mutant was higher than that of the wild type (Supplemental Fig. S5B). These findings concur with those of previous studies showing that FLV1/3-mediated AEF can oxidize P700 (Helman et al., 2003; Allahverdiyeva et al., 2013; Hayashi et al., 2014). The suppression of photosynthetic linear electron flow caused by CO₂ limitation induced P700 oxidation in S. 6803 even in the absence of...
FLV-mediated electron flow (Supplemental Fig. S5D). The oxidized P700 was reduced by the activation of FLV2/4-mediated AEF or by the resumption of photosynthetic CO₂ fixation (Supplemental Fig. S5).

S. 7942 wild type lacks FLV2/4-mediated AEF (Hayashi et al., 2014), so its PSII and PSI photosynthetic parameters responded to CO₂ limitation in almost the same manner as did those of S. 6803 Δflv4. For the S. 7942 wild type, both Y(I) and Y(II) decreased and remained low under CO₂ limitation, but they were restored by adding NaHCO₃ (Supplemental Fig. S7A). The increase in Y(ND) reflected P700 oxidation in response to CO₂ limitation and was observed in both the wild type and Δflv1/3 of S. 7942 (Supplemental Fig. S7). The mutant of S. 7942 also had a higher Y(ND) than did the S. 7942 wild type under CO₂ limitation (Supplemental Fig. S7B). These results align with the findings of a previous study (Shaku et al., 2015).

**DISCUSSION**

Table II summarizes the findings of previous studies and this study and shows two main conclusions: (1) P700 oxidation is linked directly to the protection of PSI against photoinhibition; and (2) in cyanobacteria, there are several strategies, including FLV, to alleviate PSI photoinhibition. In S. 7002, FLV1/3 mediates oxygen-dependent AEF that regulates the PSI redox state and promotes P700 oxidation under CO₂ limitation (Fig. 3; Supplemental Fig. S4; Shimakawa et al., 2016). In S. 7002, the lack of FLV-mediated AEF resulted in P700 reduction, photosynthesis suppression, PSI photoinhibition, and growth retardation (Figs. 1 and 2). These observations correspond to higher transcript levels of flv1/3 under CO₂ limitation (Ludwig and Bryant, 2012). In contrast, S. 6803 and S. 7942 keep P700 oxidized under CO₂ limitation independently of FLV-mediated AEF (which protects PSI against photooxidative damage; Supplemental Figs. S2, S5, and S7; Zhang et al., 2009; Shaku et al., 2015). In cyanobacteria, FLV has diverse physiological significance as the agent for oxygen-dependent AEF.

In this study, we showed that P700 oxidation protects PSI against photoinhibition in cyanobacteria (which are the progenitors of oxygenic phototrophs). Photooxidative damage in PSI is caused by ROS generated by excitation energy transfer from P700 ultimately to oxygen. Therefore, photooxidizable P700 in PSI can produce ROS, whereas oxidized P700 cannot be excited by photon energy. PSI photoinhibition is caused by O₂⁻ produced on the acceptor side of PSI when NADP⁺ regeneration is limited (Hihara and Sonoike, 2001). Added hydrogen peroxide reacts with reduced iron in iron-sulfur centers to form hydroxyl radicals that destroy PSI instantaneously (Hihara and Sonoike, 2001; Sonoi, 2011). P700 oxidation is expected to negate the effect of hydroxyl radicals by suppressing O₂⁻ generation and by oxidizing the iron-sulfur centers (Sonoike 1996). Recently, it was suggested that O₂⁻ triggers PSI photoinhibition (Cazzaniga et al., 2012, 2016; Takagi et al., 2016). Keeping P700 oxidized should help suppress O₂⁻ generation. P700 oxidation alleviates PSI photoinhibition in sunflower (Helianthus annuus) leaves during repetitive short saturated-pulse treatment (Sejima et al., 2014). There may be a mechanism common both to plants and cyanobacteria for protecting PSI from photooxidative damage. P700 oxidation would be a hedge against ROS generation.

In S. 6803 and S. 7942, P700 remained oxidized under CO₂ limitation even without FLV1/3 and FLV2/4 (Supplemental Figs. S5 and S7). There is, therefore, a P700 oxidation mechanism that operates independently of FLV-mediated AEF under CO₂ limitation. One candidate is cyclic electron flow around PSI, which helps induce the proton gradient across the thylakoid membrane (Miyake et al., 2004, 2005). Acidification of the luminal side reduces the oxidation activity of plastocyanin in the Cyt b₆/f complex and limits the electron flux from plastocyanin to P700 through plastocyanin (or Cyt c). These hypotheses are supported by the fact that Y(I) is greater than Y(II) for all cyanobacterial strains tested except for S. 7002 Δflv1/3 (Fig. 3; Supplemental Figs. S5 and S7). Nevertheless, we also found that Y(I) is greater than Y(II) for both S. 6803 wild type and its mutant ΔndhD1/2 (Supplemental Fig. S3). Therefore, reduced activity of NAD(P)H dehydrogenase-mediated cyclic electron flow (Ohkawa et al., 2000) is not linked to the ratio of Y(I) to Y(II). Moreover, cyclic electron transport rates in S. 6803 and S. 7002 are negligible relative to their photosynthetic linear and

<table>
<thead>
<tr>
<th>Cyanobacteria</th>
<th>Type</th>
<th>Growth</th>
<th>Y(I)</th>
<th>Y(II)</th>
<th>P700 Redox</th>
<th>PSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. 7002</td>
<td>Wild type</td>
<td>Well</td>
<td>High</td>
<td>High</td>
<td>Oxidized</td>
<td>Safe</td>
</tr>
<tr>
<td></td>
<td>Δflv1/3</td>
<td>Bad</td>
<td>Low</td>
<td>Low</td>
<td>Reduced</td>
<td>Broken</td>
</tr>
<tr>
<td>S. 6803</td>
<td>Wild type</td>
<td>Well</td>
<td>High</td>
<td>High</td>
<td>Oxidized</td>
<td>Safe</td>
</tr>
<tr>
<td></td>
<td>Δflv1/3</td>
<td>Well</td>
<td>High</td>
<td>High</td>
<td>Oxidized</td>
<td>Safe</td>
</tr>
<tr>
<td></td>
<td>Δflv4</td>
<td>Bad</td>
<td>Low</td>
<td>Low</td>
<td>Oxidized</td>
<td>Safe</td>
</tr>
<tr>
<td></td>
<td>Δflv1/3/4</td>
<td>–</td>
<td>Low</td>
<td>Low</td>
<td>Oxidized</td>
<td>Safe</td>
</tr>
<tr>
<td>S. 7942</td>
<td>Wild type</td>
<td>Well</td>
<td>Low</td>
<td>Low</td>
<td>Oxidized</td>
<td>Safe</td>
</tr>
<tr>
<td></td>
<td>Δflv1/3</td>
<td>Well</td>
<td>Low</td>
<td>Low</td>
<td>Oxidized</td>
<td>Safe</td>
</tr>
</tbody>
</table>


Table II. Phenotypes of each wild type and flv mutant under CO₂ limitation in three cyanobacteria species used in this study.
were measured with a spectrophotometer (U-2800A; Hitachi). For all photo-
harvested and resuspended by vortexing in 1 mL of 100% (v/v) methanol. After
this, the electron flow from the Cyt b6/f complex to P700 in PSI decreases. This
response is called RISE (Shaku et al., 2015), and it
might be the main driver of P700 oxidation under CO2
limitation in cyanobacteria. Respiratory terminal oxi-
dases like Cyt c oxidase and cytochrome bd-type quinol
oxidase also may contribute to the oxidation of the donor
side of PSI under CO2 limitation (Beardall et al., 2003;
Trouillard et al., 2012; Lea-Smith et al., 2013). It is dif
ficult, however, to explain why Y(II) decreased during
CO2 limitation in the cyanobacteria we studied (Fig. 3;
Supplemental Figs. S5 and S7).

In S. 6803, FLV2/4 may receive electrons from the
acceptor side of PSI. In both S. 6803 wild type and its
mutant Δflv1/3, the increases in Y(I) and Y(II) indicate that
PSI electron flux is restored under CO2 limitation (Supplemental Fig. S5, A and B). Unlike S. 6803 wild
type and Δflv1/3, neither Δflv4 nor Δflv1/3/4 experienced an increase in Y(I) (Supplemental Fig. S5, C and D). Removing oxygen lowered Y(II) under CO2
limitation in S. 6803 wild type but not in Δflv4 (Supplemental Fig. S6). These data suggest that FLV2/4 mediates an oxygen-dependent AEf on the acceptor side of PSI (Hayashi et al., 2014; Shimakawa et al., 2015), as does FLV1/3 (Helman et al., 2003), since both FLV sets have similar primary structures (Fujisawa et al., 2014) and enzymatic characteristics of recombinant proteins (Vicente et al., 2002; Shimakawa et al., 2015). However, we cannot exclude the possibility that the relief of ex-
citation pressure at PSI by FLV2/4 (Bersanini et al., 2014) provides an enhancement of Y(I) during CO2-
limited photosynthesis in S. 6803. FLV2/4 is known to interact with PSI1 and phycobilisomes (Bersanini et al.,
2014), so in S. 6803, it may have multiple functions to alleviate photoinhibition under low CO2.

**MATERIALS AND METHODS**

**Growth Conditions and Chl a Determination**

Cyanobacterial cultures were maintained under continuous fluorescent
lighting (25°C, 50 μmol photons m−2 s−1) on BG-11 solid medium (for Synechocystis sp. PCC 6803 and Synechococcus elongatus PCC 7942) and A’-solid
medium (for Synechococcus sp. PCC 7002; Allen, 1968; Stevens and Porter, 1980).
Cells from both cultures were inoculated into liquid medium (initial OD750 =
0.1–0.2) and grown on a rotary shaker (100 rpm) under continuous fluorescent
lighting (25°C, 150 μmol photons m−2 s−1) at 2,000 μL L−1 (CO2). OD678 values
were measured with a spectrophotometer (U-2800A; Hitachi). For all photo-
synthetic parameter measurements, cells from the exponential growth phase
were used. In the experiments for Figure 1 and Supplemental Figure S4, S. 7002
was grown under ambient [CO2].

For Chl measurements, cells from 0.1- to 1-mL cultures were centrifugally
harvested and resuspended by vortexing in 1 mL of 100% (v/v) methanol. After
incubation at room temperature for 5 min, the suspension was centrifuged at
10,000g for 5 min. Total Chl a was spectrophotometrically determined from the
supernatant (Grime and Boardman, 1972).

**Bioinformatics**

All the S. 7002, S. 6803, and S. 7942 gene sequence data used in this study
were obtained from Cyanobase (http://genome.microbedb.jp/CyanoBase; Fujiwara et al., 2014). For the flv1–4, cox, cyd, arito, mlhD1/2, and pgr5 gene se-
quences, BLAST searches were conducted in Cyanobase.

**Statistical Analysis**

Student’s t tests were applied to detect differences. All statistical analy-
ses were performed using Microsoft Excel 2010 (Microsoft) and JMP8 (SAS
Institute).

**Generation of Mutants**

The triple mutant of S. 6803 deficient in flv1 (dfl1521), flv3 (dfl3505), and
flv4 (dfl2017) was generated by transforming Δflv1/3 (Hayashi et al., 2014) using the
flv4 construct (Shimakawa et al., 2013). PCR was used to confirm the complete
segregation of flv1 and flv4 (Supplemental Fig. S1A). The disruption of FLV3
proteins was verified by immunoblotting with a specific antibody to FLV3 (see “Immunoblot Analysis” below), since a nonspecific band was observed near the
target band in the PCR analysis (Supplemental Fig. S1B).

To construct the double mutant of S. 7002 lacking flv1 (SYNPPCC7002_A1743)
and flv3 (SYNPPCC7002_A1321) orthologs, PCR was used to amplify each ge
nome region encoding A1743 and A1321 with the up f and dn r primer sets
(Supplemental Table S1). They were then cloned into the pGEM-T Easy vector
(Promega). The recombinant plasmids containing A1743 and A1321 were lin-
earized and amplified by inverse PCR with the up f and dn r primer sets
(Supplemental Table S1). They were then applied to the In-Fusion reaction
(Takara) using chloramphenicol and kanamycin resistance genes (“Cmr” and
“Kan”) derived from pACYC184 and pUC4K vectors, respectively (Rose, 1988;
Taylor and Rose, 1988). Transformation of S. 7002 was performed by the
standard procedure (Frigaard et al., 2004). Single mutants (Δflv1 and Δflv3) were
selected on 0.5% BG-11 agar plates containing chloramphenicol (15 μg mL−1)
or kanamycin (50 μg mL−1). The double mutant (Δflv1/3) was generated by trans-
forming Δflv1 with the Δflv3 construct. The mutants were selected on plates
containing both chloramphenicol (15 μg mL−1) and kanamycin (50 μg mL−1).
Complete segregation was confirmed by PCR (Supplemental Fig. S2C).

**Immunoblot Analysis**

S. 6803 wild-type and Δflv1/3/4 cell cultures (10 mL) were harvested by
centrifugation and pellet resuspension in 500 μL of extraction buffer (50 mM
HEPES-KOH [pH 7.5], 1 mM MgCl2, 2 mM EDTA, and 1 mM phenyl-
methylsulfonyl fluoride). The suspensions were homogenized with glass beads
using Bug Crusher GM-01 (Takelite) and centrifuged at 13,000g for 30 min at 4°C.
The supernatants were treated as extracted soluble fractions. Protein concen-
trations in them were determined with the Pierce 660 nm Protein Assay
(Thermo Scientific) using bovine serum albumin as the standard. Soluble
fractions containing 5 μg of protein were analyzed by SDS-PAGE. After elec-
trophoresis, the proteins were electrotransferred to a polyvinylidene fluoride
membrane and detected by an FLV3-specific antibody (kindly provided by Dr.
H. Yamamoto).

**Measurement of Chl Fluorescence and P700**

Chl fluorescence and P700 were measured simultaneously with the Dual-
PAM-100 system (Heinz Walz) at room temperature (25°C ± 2°C). For S. 6803
and S. 7942, the reaction mixtures (2 mL) contained 50 mM HEPES (pH7.5) and
the cells (10 μg Chl mL−1). For S. 7002, the reaction mixture consisted of fresh A’-
medium and the cells (10 μg Chl mL−1). During the measurements, the reaction
mixtures were stirred with a magnetic micro stirrer. The photon flux densities of
red AL (LED with peak emission at 635 nm) are shown in the corresponding
figure legends. Y(II) reflects the apparent electron flux in photosynthetic linear
electron transport (Genty et al., 1989). It was calculated from Chl
fluorescence as (Fm′ - F0) / Fm′ (where Fm′ = maximum variable fluorescence yield, F0 = steady-
state fluorescence yield, and Fm′ = minimum fluorescence yield; Schreiber et al.,
2014).
1986; van Kooten and Snel, 1990). The redox state of P700 was determined according to the method of Klughammer and Schreiber (1994, 2008). In this procedure, \( P_m \) was the maximum P700 photooxidation level obtained by saturated pulse light under far-red illumination, \( P = \) the oxidation level of P700 under AL, \( P_m' = \) maximum oxidation level of P700 obtained by saturation pulse under AL illumination, \( Y(\text{P}) = (P_{m'} - P)/P_m = \) the incident quantum yield of photochemical energy conversion, \( Y(\text{ND}) = P/P_m = \) the quantum yield of non-photochemical energy dissipation due to donor-side limitation, and \( Y(\text{NA}) = (P_{m'} - P_m)/P_m = \) the quantum yield of nonphotochemical energy dissipation due to acceptor-side limitation. The sum of the three factors \( Y(I) = Y(\text{ND}) + Y(\text{NA}) = 1 \). For the simultaneous measurements of \( Y(I), Y(I), Y(\text{ND}), \) and \( Y(\text{NA}), \) a 300-ms saturation pulse (10,000 \( \mu \)mol photons m \(^{-2}\) s \(^{-1}\)) was supplied every 10 min. The stirrer was turned off 5 s before the saturation pulse was applied.

**Measurement of Oxygen Exchange**

Oxygen uptake and evolution were measured with a Clark-type oxygen electrode (Hansatech; Shimakawa et al., 2015). For S. 6803 and S. 7942, the reaction mixture (2 mL) contained 50 mM HEPES (pH 7.5), 10 mM NaHCO\(_3\), and the cells (10 \( \mu \)g Chl mL \(^{-1}\)). For S. 7002, the mixture (2 mL) contained fresh A. medium, 10 mM NaHCO\(_3\), and the cells (10 \( \mu \)g Chl mL \(^{-1}\)). Cells were illuminated with AL (red light, 620 \( \mu \)mol photons m \(^{-2}\) s \(^{-1}\)) and the mutant \( \Delta \text{fi} \), \( \Delta \text{fi} \) wavelength 695 nm; photon flux density of Chl \( a \) was measured with a Schott RG9 long-pass filter. The fluorescence terminology follows van Kooten and Snel (1990).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Insertional inactivation of \( f o \) genes in S. 6803 and S. 7002.

**Supplemental Figure S2.** Decreased activities of PSI and photosynthesis in the wild type and the \( f o \) mutants of S. 6803 and S. 7942 after 2-h exposure to C\(_2\)O limited during illumination.

**Supplemental Figure S3.** Responses of the photosynthetic parameters of PSI and PSII to C\(_2\)O limitation in the mutant of S. 6803 deficient in \( ndhD1 \) and \( ndhD2 \).

**Supplemental Figure S4.** Photosynthetic parameters of S. 7002 wild type and \( \Delta \text{fi} \) grown in ambient [CO\(_2\)].

**Supplemental Figure S5.** Responses of the photosynthetic parameters of PSII and PSI to CO\(_2\) limitation in S. 6803 wild type and the mutants \( \Delta \text{fi} \), \( \Delta \text{fi} \) and \( \Delta \text{fi} \).

**Supplemental Figure S6.** Effects of eliminating oxygen on the photosynthetic parameters of PSI and PSII under C\(_2\)O limitation in S. 6803 wild type and the mutant \( \Delta \text{fi} \).

**Supplemental Figure S7.** Responses of the photosynthetic parameters of PSI and PSII to CO\(_2\) limitation in S. 7942 wild type and the mutant \( \Delta \text{fi} \).

**Supplemental Table S1.** Primers used in this study.

**ACKNOWLEDGMENTS**

We thank Akihiko Kondo, Tomohisa Hasunuma, and Dr. Shimpei Aikawa (Kobe University) for supplying the S. 7002 wild type; Dr. Hiroshi Ohkawa (Hiroshima University) and Kintake Sonoko (Waseda University) for giving us the mutant \( \Delta \text{nahL} \); Dr. Hiroshi Yamamoto (Kyoto University) for the gift of the anti-FvLY3 antibody; and Editage (www.editage.jp) for English language editing.

Received August 4, 2016; accepted September 7, 2016; published September 9, 2016.

**LITERATURE CITED**


Allen MM (1968) Simple conditions for growth of unicellular blue-green algae on plates. 2. J Physiol 4: 1–4


Grimme LH, Boardman NK (1972) Photochemical activities of a particle fraction P 1 obtained from the green alga Chlorella fusca. Biochem Biophys Res Commun 49: 1617–1623


Fig. S1. Insertional inactivation of *flv* genes in S. 6803 and S. 7002. (A) DNA fragments amplified by PCR showing complete segregation of the inactivated genes, *flv1* (*sll1521*) and *flv4* (*sll0217*). WT, S. 6803 WT; Δt, the triple mutant of S. 6803 (Δ*flv1/3/4*). (B) Western blot analysis showing the lack of the *flv3* (*sll0550*) gene product. Extracted soluble fractions (5 µg protein/lane) of S. 6803 WT and Δ*flv1/3/4* were analyzed. (C) DNA fragments amplified by PCR showing complete segregation of the inactivated genes, *flv1* (*SYNPCC7002_A1743*) and *flv3* (*SYNPCC7002_A1321*). WT, S. 7002 WT; Δs, single mutants of S. 7002 (Δ*flv1* and Δ*flv3*); Δd, the double mutant of S. 7002 (Δ*flv1/3*).
Fig. S2. Decreased activities of PSI and photosynthesis in the WT and the flv mutants of S. 6803 (A, C, E) and S. 7942 (B, D, F) after 2-hour exposures to CO$_2$ limitation during illumination (290 µmol photons m$^{-2}$ s$^{-1}$). Reaction mixtures contained 50 mM HEPES (pH 7.5) and cells (10 µg Chl mL$^{-1}$). Residual total oxidizable P700 (A, B) and photosynthetic O$_2$-evolution rates (C–F) were measured before and after 1 h in the dark following treatments. Black and red symbols represent each WT and flv mutant, respectively. Closed and open symbols represent the data before and after the treatments (C–F), respectively. Photosynthetic O$_2$-evolution rates were measured in the presence of 10 mM NaHCO$_3$. Each measurement was conducted three times, and the means ± SD are shown.

Fig. S2. Shimakawa et al.
Fig. S3. Responses of the photosynthetic parameters of PSII and PSI to CO₂ limitation in the mutant of S. 6803 deficient in *ndhD1* and 2 (*ΔndhD1/2*). Reaction mixtures contained the cells (10 µg Chl mL⁻¹). (A) Time courses of the parameters of PSII and PSI. Y(I), black circles; Y(ND), red triangles; Y(NA), blue squares; Y(II), white diamonds. Red AL (180 µmol photons m⁻² s⁻¹) was activated at time zero. NaHCO₃ (10 mM) was added as indicated. Measurements were conducted three times, and representative data are shown. (B) The relationship between Y(II) and Y(I) throughout the measurement in S. 6803 WT (Black circles) and *ΔndhD1/2* (green stars). Data plotted are obtained from Figs. 3A and S2A, respectively.
Fig. S4. Photosynthetic parameters of S. 7002 WT and Δflv1/3 grown in ambient [CO₂]. (A, B) Time courses of dissolved O₂ concentration (red lines) and relative Chl fluorescence yield (black lines) in S. 7002 WT (A) and Δflv1/3 (B). Experimental procedures were similar to those in our recent study (Shimakawa et al., 2016). Cells (10 μg Chl mL⁻¹) were illuminated with measuring light (ML) as indicated. Red AL (300 μmol photons m⁻² s⁻¹, 620 < λ < 695 nm) was activated at time zero. Dashed arrows indicate changes in the position of the Chl fluorescence signal. Chl fluorescence parameters are: F₀, minimum fluorescence under ML; Fₛ, steady-state fluorescence under AL; Fₘ', maximum variable fluorescence under saturating light. NaHCO₃ (10 mM) was added as indicated. Experiments were performed three times, and representative data are shown. (C) Time courses of Y(II) shown as mean ± SD (n = 3). Black circles, S. 7002 WT; red triangles, Δflv1/3. (D) Relationships between gross O₂ evolution rates and Y(II) in S. 7002 WT (black circles) and Δflv1/3 (red triangles). Photosynthetic O₂ evolution rates were determined at both CO₂-saturated and CO₂-limited conditions in separate experiments (n = 3) following the methods described in Shimakawa et al. (2016).
Fig. S5. Responses of the photosynthetic parameters of PSII and PSI to CO$_2$ limitation in S. 6803 WT (A) and the mutants Δflv1/3 (B), Δflv4 (C) and Δflv1/3/4 (D). Reaction mixtures contained the cells (10 µg Chl mL$^{-1}$). Y(I), black circles; Y(ND), red triangles; Y(NA), blue squares; Y(II), white diamonds. Red AL (180 µmol photons m$^{-2}$ s$^{-1}$) was activated at time zero. NaHCO$_3$ (10 mM) was added as indicated. Measurements were conducted three times and representative data are shown.
**Fig. S6.** Effects of eliminating O$_2$ on the photosynthetic parameters of PSII and PSI under CO$_2$ limitation in S. 6803 WT (A) and the mutant Δflv4 (B). Reaction mixtures contained the cells (10 µg Chl mL$^{-1}$). Y(I), black circles; Y(ND), red triangles; Y(NA), blue squares; Y(II), white diamonds. Red AL (180 µmol photons m$^{-2}$ s$^{-1}$) was activated at time zero. Glucose (5 mM), catalase (250 units mL$^{-1}$), and glucose oxidase (GlcOX, 5 units mL$^{-1}$) were added as indicated. Measurements were conducted three times and representative data are shown.
**Fig. S7.** Responses of the photosynthetic parameters of PSII and PSI to CO$_2$ limitation in S. 7942 WT (A) and the mutant Δflv1/3 (B). Reaction mixtures contained the cells (10 µg Chl mL$^{-1}$). Y(I), black circles; Y(ND), red triangles; Y(NA), blue squares; Y(II), white diamonds. Red AL (180 µmol photons m$^{-2}$ s$^{-1}$) was activated at time zero. NaHCO$_3$ (10 mM) was added as indicated. Measurements were conducted three times and representative data are shown.
### Supplemental Table S1. Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences (5’−3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1743 up f</td>
<td>CTGGGATTCGAAACACATTTT</td>
</tr>
<tr>
<td>A1743 up r</td>
<td>TCTTACGTGCCGATCCGATCCAGTGCGGCTAGTTAT</td>
</tr>
<tr>
<td>A1743 dn f</td>
<td>TGACCCTGCTTCTACGCTGACCCACGAGAAATTA</td>
</tr>
<tr>
<td>A1743 dn r</td>
<td>GCATAGATCCACCAATGGTCA</td>
</tr>
<tr>
<td>A1321 up f</td>
<td>ATCCAGACAGAAAAGGTTAACGAC</td>
</tr>
<tr>
<td>A1321 up r</td>
<td>AAACCGCCCAGTCTACCATAATCAGAGACGTAAATCCCG</td>
</tr>
<tr>
<td>A1321 dn f</td>
<td>GTTGGGCTTCGGAATGGAGTTGCTCCGTAGAAATGGTC</td>
</tr>
<tr>
<td>A1321 dn r</td>
<td>CGCAGCGACTTTGCTATACAC</td>
</tr>
</tbody>
</table>