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FLAVODIIRON2 and FLAVODIIRON4 Proteins Mediate an Oxygen-Dependent Alternative Electron Flow in Synechocystis sp. PCC 6803 under CO₂-Limited Conditions

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This study aims to elucidate the molecular mechanism of an alternative electron flow (AEF) functioning under suppressed (CO₂-limited) photosynthesis in the cyanobacterium Synechocystis sp. PCC 6803. Photosynthetic linear electron flow, evaluated as the quantum yield of photosystem II [Y(II)], reaches a maximum shortly after the onset of actinic illumination. Thereafter, Y(II) transiently decreases concomitantly with a decrease in the photosynthetic oxygen evolution rate and then recovers to a rate that is close to the initial maximum. These results show that CO₂ limitation suppresses photosynthesis and induces AEF. In contrast to the wild type, Synechocystis sp. PCC 6803 mutants deficient in the genes encoding FLAVODIIRON2 (FLV2) and FLV4 proteins show no recovery of Y(II) after prolonged illumination. However, Synechocystis sp. PCC 6803 mutants deficient in genes encoding proteins functioning in photorespiration show AEF activity similar to the wild type. In contrast to Synechocystis sp. PCC 6803, the cyanobacterium Synechococcus elongatus PCC 7942 has no FLV proteins with high homology to FLV2 and FLV4 in Synechocystis sp. PCC 6803. This lack of FLV2/4 may explain why AEF is not induced under CO₂-limited photosynthesis in S. elongatus PCC 7942. As the glutathione S-transferase fusion protein overexpressed in Escherichia coli exhibits NADH-dependent oxygen reduction to water, we suggest that FLV2 and FLV4 mediate oxygen-dependent AEF in Synechocystis sp. PCC 6803 when electron acceptors such as CO₂ are not available.

In photosynthesis, photon energy absorbed by PSI and PSII in thylakoid membranes oxidizes the reaction center chlorophylls (Chls), P700 in PSI and P680 in PSII, and drives the photosynthetic electron transport (PET) system. In PSI, water is oxidized to oxygen as the oxidized P680 accepts electrons from water. These electrons then reduce the cytochrome b₆/f complex through plastocyanine (PQ) in the thylakoid membranes. Photooxidized P700 in PSI accepts electrons from the reduced cytochrome b₆/f complex through plastocyanin or cytochrome c₆. Electrons released in the photooxidation of P700 are used to produce NADPH through ferredoxin and ferredoxin NAD⁺ reductase. Thus, electrons flow from water to NADPH in the so-called photosynthetic linear electron flow (LEF). Importantly, LEF induces a proton gradient across the thylakoid membranes, which provides the driving force for ATP production by ATP synthases in the thylakoid membranes. NADPH and ATP serve as chemical energy donors in the photosynthetic carbon reduction cycle (Calvin cycle).

It recently has been proposed that, in cyanobacteria, the photorespiratory carbon oxidation cycle (photorespiration) functions simultaneously with the Calvin cycle to recover carbon for the regeneration of ribulose-1,5-bisphosphate, one of the substrates of Rubisco (Hagemann et al., 2013). Rubisco catalyzes the primary reactions of carbon reduction as well as oxidation cycles. However, the presence of a specific carbon concentration mechanism (CCM) in cyanobacteria had been thought to prevent the operation of photorespiration. CCM maintains a high concentration of CO₂ around Rubisco so that the oxygenase activity of Rubisco is suppressed (Badger and Price, 1992). However, recent studies on mutants deficient in...
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photorespiration enzymes have shown that photorespiration functions, particularly under CO₂-limited conditions, in cyanobacteria as it does in higher plants (Eisenhut et al., 2006, 2008).

Decreased consumption of NADPH under CO₂-limited or high-light conditions causes electrons to accumulate in the PET system. As a result, the photokiss and photoreduction cycles of the reaction center Chls in PSI and PSII become uncoupled from the production of NADPH, inducing alternative electron flow (AEF) pathways (Mullineaux, 2014). In cyanobacteria, several AEFs that differ from those in higher plants are proposed to function as electron sinks (Mullineaux, 2014). Electrons accumulated in the PET system flow to oxygen through FLAVODIIRON1 (FLV1) and FLV3 proteins in PSI and the terminal oxidase, cytochrome c oxidase complex, and cytochrome bd-quinol oxidase (Pils and Schmetterer, 2001; Berry et al., 2002; Helman et al., 2003; Nomura et al., 2006; Lea-Smith et al., 2013). Cyanobacterial FLV comprises a diiron center, a flavodoxin domain with an FMN-binding site, and a flavin reductase domain (Vicente et al., 2002). In Synechocystis sp. PCC 6803, Helman et al. (2003) identified four genes encoding FLV1 to FLV4 and showed that FLV1 and FLV3 were essential for the photoreduction of oxygen by PSI. FLV1 and FLV3 were proposed to function as a heterodimer (Allahverdiyeva et al., 2013). FLV2/4 have been proposed to function in energy dissipation associated with PSII (Zhang et al., 2012). In addition, hydrogenases convert H⁺ to H₂ with NADPH as an electron donor (Appel et al., 2000). Furthermore, Flores et al. (2005) suggested that the nitrate assimilation pathway functions in AEF when the cells live in medium containing nitrate.

To elucidate the physiological functions of these AEFs, evaluation of the presence and capacity of each AEF pathway is required. Therefore, in vivo analyses of electron fluxes are essential. We had found that an electron flow uncoupled from photosynthetic oxygen evolution functioned under suppressed (CO₂-limited) photosynthesis in the cyanobacterium Synechocystis sp. PCC 6803 but not in Synechococcus elongatus PCC 7942 (Hayashi et al., 2014), indicating that an AEF operated in Synechocystis sp. PCC 6803. This AEF was induced in high-[CO₂]-grown Synechocystis sp. PCC 6803 during the transition from CO₂-saturated photosynthesis to CO₂-limited photosynthesis (Hayashi et al., 2014). In contrast, in Synechocystis sp. PCC 6803 grown at ambient CO₂ concentration, AEF was detected immediately following the transition to CO₂-limited photosynthesis (Hayashi et al., 2014), suggesting that AEF was already induced under ambient atmospheric conditions.

The expression of the AEF activity observed under CO₂-limited photosynthesis required the presence of oxygen in Synechocystis sp. PCC 6803 (Hayashi et al., 2014). In Synechocystis sp. PCC 6803, FLV1/3 were proposed to catalyze the photoreduction of oxygen (Helman et al., 2003). However, Hayashi et al. (2014) found no evidence that FLV1/3 operated under CO₂-limited photosynthesis: a mutant Synechocystis sp. PCC 6803 deficient in FLV1/3 maintained almost constant electron flux under CO₂-limited photosynthesis after the transition from CO₂-saturated conditions. Thus, the postulated photoreduction of oxygen by FLV1/3 was not responsible for the electron flux observed under CO₂-limited photosynthesis in Synechocystis sp. PCC 6803.

In this study, we aimed to elucidate the molecular mechanism of the oxygen-dependent AEF functioning under CO₂-limited photosynthesis in Synechocystis sp. PCC 6803. The possibility that FLV2 and FLV4 catalyze the photoreduction of oxygen under CO₂-limited photosynthesis could not be excluded, given that AEF in high-[CO₂]-grown Synechocystis sp. PCC 6803 was induced following the transition to CO₂-limited photosynthesis (Hayashi et al., 2014). Both FLV2 and FLV4 are predicted to possess oxidoreductase motifs, similar to FLV1 and FLV3 (Helman et al., 2003; Zhang et al., 2012). Furthermore, the expression of two FLV genes (flv2 and flv4) was enhanced under low-[CO₂] conditions (Zhang et al., 2009). Zhang et al. (2012) proposed that FLV2 and FLV4 did not donate electrons to oxygen on the basis of the finding that the Synechocystis sp. PCC 6803 mutants deficient in FLV1/3 showed no light-dependent oxygen uptake (Helman et al., 2003). However, Helman et al. (2003) cultivated Synechocystis sp. PCC 6803 strains deficient in FLV1 and FLV3 proteins under high-[CO₂] conditions, and we cannot exclude the possibility that the FLV2 and FLV4 proteins were not produced in the studied cells. Taken together, it seems plausible that FLV2 and FLV4 mediate oxygen-dependent AEF following the transition to CO₂-limited photosynthesis. To evaluate this possibility, we constructed Synechocystis sp. PCC 6803 mutants deficient in flv2 and flv4 and measured their oxygen evolution and Chl fluorescence simultaneously. The mutants showed suppressed LEF after transition to CO₂-limited photosynthesis, similar to S. elongatus PCC 7942. We also tested the possibility that photorespiration functions as an electron sink under CO₂-limited photosynthesis in Synechocystis sp. PCC 6803. A recent study revealed photorespiratory oxygen uptake in a flv1/3 mutant under CO₂-depleted conditions (Allahverdiyeva et al., 2011). In this study, we found that the quantum yield of photosystem II [Y(II)] of mutants deficient in genes encoding proteins that function in photorespiration was similar to that of wild-type Synechocystis sp. PCC 6803. Thus, FLV2 and FLV4 appear to function in the oxygen-dependent AEF under CO₂-limited photosynthesis in Synechocystis sp. PCC 6803. This inference is further supported by the lack of FLV2 and FLV4 homologs in the genome of S. elongatus PCC 7942 (Bersanini et al., 2014). In addition, we found oxygen-reducing activities of recombinant glutathione S-transferase (GST)-FLV4 fusion protein, similar to those of recombinant FLV3 protein (Vicente et al., 2002). In light of these results, we discuss the molecular mechanism of the oxygen-dependent AEF under CO₂-limited photosynthesis and the physiological function of FLV proteins in Synechocystis sp. PCC 6803.
RESULTS

FLV2 and FLV4 Are Essential for AEF under CO₂-Limited Photosynthesis in *Synechocystis* sp. PCC 6803

We monitored Chl fluorescence and the concentration of oxygen in medium containing *Synechocystis* sp. PCC 6803 and *S. elongatus* PCC 7942 cells (Fig. 1). During the measurements, the top of the reaction chamber was open to allow equilibration of the medium with air. Cyanobacteria were added after oxygen concentration in the medium had equilibrated with the concentration in air (about 250 μM at 25°C) in the dark. With both of the cyanobacterial strains (compare Fig. 1, A and C), the medium oxygen concentration quickly declined to a lower equilibrium due to respiratory oxygen consumption. Upon illumination with actinic light (200 μmol photons m⁻² s⁻¹), the relative Chl fluorescence increased from minimum to steady state and then decreased transiently (Fig. 1, A and C), while the electron flux in PSII \( Y(II) = (F_m' - F_s)/F_m' \), where \( F_m' \) is maximum variable fluorescence and \( F_s \) is steady-state fluorescence under actinic light] was high (Fig. 1, B and D). Actinic light started photosynthesis as indicated by the increase in oxygen concentration in the medium (Fig. 1, A and C). In both strains, medium oxygen concentration reached a peak when \( F_s \) had started to increase; \( F_s \) continued to rise while \( Y(II) \) decreased and oxygen concentration shifted toward a lower equilibrium (*Synechocystis* sp. PCC 6803, Fig. 1, A and B; *S. elongatus* PCC 7942, Fig. 1, C and D). Thereafter, the parameters developed differently in the two strains. \( F_s \) decreased continuously, paralleled by an increasing \( Y(II) \) in *Synechocystis* sp. PCC 6803 (Fig. 1, A and B). In contrast, \( F_s \) and \( Y(II) \) remained at high and low levels, respectively, in *S. elongatus* PCC 7942 (Fig. 1, C and D). Addition of CO₂ (as NaHCO₃) caused an increase in medium oxygen concentration and a decrease in \( F_s \) in both strains (Fig. 1, A and C) as well as a sharp increase of \( Y(II) \) in *S. elongatus* PCC 7942 (Fig. 1D). In separate experiments, we determined oxygen evolution rates at the three times marked in Figure 1 as \( \alpha \) (CO₂ in the medium not yet depleted by photosynthesis), \( \beta \) (medium CO₂ at a low steady state controlled by

![Figure 1](https://www.plantphysiol.org/)

*Figure 1*. Development of photosynthetic parameters in media containing high-[CO₂]-grown *Synechocystis* sp. PCC 6803 (A and B) and *S. elongatus* PCC 7942 (C and D). A and C. Time courses of medium oxygen concentration (red traces) and relative Chl fluorescence (black traces). Initially, media containing cyanobacteria (10 μg Chl mL⁻¹) were kept dark; illumination with measuring light (ML) and actinic light (AL; 200 μmol photons m⁻² s⁻¹) started as indicated. The downward shift in the Chl fluorescence signal (\( F_o \)) after the start of ML illumination is due to increasing the instrument amplification from 200 to 100 mV. Chl fluorescence parameters are as defined in the text. NaHCO₃ (final concentration, 10 mM) was added as indicated. \( \alpha \), \( \beta \), and \( \gamma \) denote times at which oxygen evolution rates were determined in separate experiments. From about the time of maximum oxygen concentration to the addition of NaHCO₃, photosynthesis can be considered CO₂ limited. The experiments were performed four times, and representative data are shown. B and D. Time courses of \( Y(II) \), calculated for the corresponding measurements of \( F_m' \) as \( (F_m' - F_s)/F_m' \). Values shown are means ± SD (n = 4).
photosynthetic CO₂ consumption and diffusion from the atmosphere), and γ (high CO₂ availability due to added NaHCO₃; compare Supplemental Fig. S1). In both strains, oxygen evolution rates were similarly high at times α and γ, indicating CO₂-saturated photosynthesis, and low at time β, indicating CO₂-limited photosynthesis (Table I). Under these conditions, the gradual drop of F₅′ and the parallel increase of Y(II) (Fig. 1, A and B) in Synechocystis sp. PCC 6803 strongly suggested the operation of an AEF under CO₂-limited photosynthesis.

F₅′ increased during the transition (Fig. 1). The increase in F₅′ could be caused by a state transition from state II to state I (McConnell et al., 2002). However, low-temperature fluorescence spectra of Synechocystis sp. PCC 6803 and S. elongatus PCC 7942 showed no changes during the transition to CO₂-limited photosynthesis (Miller et al., 1996; Hayashi et al., 2014). Thus, there is no evidence to support a state transition. The molecular mechanism for the increase in F₅′ remains unknown. Therefore, we focused on the responses of AEF to the electron sink limitation that occurred during the shift from CO₂-saturated to CO₂-limited photosynthesis.

Synechocystis sp. PCC 6803, in which the transition from CO₂-saturated to CO₂-limited photosynthesis appeared to induce AEF, has four flv genes in its genome: flv1 (sll1521), flv2 (sll0219), flv3 (sll0550), and flv4 (sll0217). The activity of isozymes FLV2 and FLV4 seems necessary for AEF under CO₂-limited photosynthesis as wild-type Synechocystis sp. PCC 6803 (Hayashi et al., 2014). Furthermore, the genes encoding FLV2 and FLV4 are absent from the genome of S. elongatus PCC 7942 (Bersanini et al., 2014), while flv1 and flv3 are present (Synpcc7942_1810, ortholog of flv1; Synpcc7942_1809, ortholog of flv3). We hypothesized that FLV2 and FLV4 are involved in the AEF under CO₂-limited photosynthesis and constructed mutants deficient in flv2 and flv4 (∆flv2 and ∆flv4, respectively; Supplemental Figs. S2 and S3). In contrast to the double mutant deficient in flv1/β (Hayashi et al., 2014), neither ∆flv2 nor ∆flv4 showed any increase in Y(II) during the transition from CO₂-saturated to CO₂-limited photosynthesis (Fig. 2, B and D), while F₅ increased and then maintained its level (Fig. 2, A and C). On addition of NaHCO₃, F₅ decreased and Y(II) recovered to values found under CO₂-saturated photosynthesis. The oxygen evolution rates in ∆flv2 and ∆flv4 decreased from time α to time β and recovered at time γ (Table I). Thus, suppression of Y(II) in ∆flv2 and ∆flv4 was due to the limited capacity of the CO₂-dependent electron sink, which could not be substituted by AEF. We concluded that in Synechocystis sp. PCC 6803, FLV2 and FLV4 are necessary for AEF under CO₂-limited photosynthesis. FLV2 and FLV4 form heterodimers (Zhang et al., 2012), and deletion of flv4 suppresses the production of FLV2 (Eisenhut et al., 2012). Thus, deficiency in either of the two genes resulted in the complete loss of physiological function (Fig. 2).

The GST-FLV4 Fusion Protein Reduces Oxygen to Water

In Synechocystis sp. PCC 6803, FLV2 and FLV4 are required for AEF under CO₂-limited photosynthesis (Fig. 2), which also depends on the presence of oxygen in the flv1/β double mutant (Hayashi et al., 2014). These facts suggested that FLV2 and FLV4 donate electrons to oxygen. To determine whether oxygen can accept electrons from FLV2 and FLV4 in vitro, we expressed recombinant GST-FLV2 and GST-FLV4 fusion proteins in Escherichia coli and tried to purify them by affinity chromatography (Supplemental Fig. S4). Unfortunately, GST-FLV2 could not be separated from a contaminant protein of about 60 to 65 kD (Supplemental Fig. S4). Therefore, we could only examine the GST-FLV4 protein in the following experiments.

GST-FLV4 catalyzed the oxidation of NADH as monitored by the decrease in A₃₃₀. The Kₘ value for NADH was approximately 30 μM (Supplemental Fig. S5), similar to that of the recombinant FLV3 protein (Vicente et al., 2002). We could not obtain Kₘ for the NADPH-dependent oxygen reduction, owing to the significantly lower activity. GST-FLV4 also reduced oxygen using NADH as the electron donor (Fig. 3). Addition of GST-FLV4 to a reaction mixture containing 50 mM Tris-HCl (pH 7.4) and 1 mM NADH stimulated the reduction of oxygen at the rate of 20.2 ± 1.5 min⁻¹ (Fig. 3). No oxygen depletion was observed when GST-FLV4 protein was added to the reaction mixture as a control. When catalase was added, no oxygen evolved, indicating that GST-FLV4 reduces oxygen to water without generating reactive oxygen. Therefore, we could only examine the GST-FLV4 protein in the following experiments.
oxygen species (Fig. 3). Furthermore, addition of dithionite showed that oxygen was completely consumed (Fig. 3). The $K_m$ value for oxygen was below 10 $\mu$M. These results demonstrate the oxygen-reducing activity of GST-FLV4, which resembles that of the recombinant FLV3 protein (Vicente et al., 2002).

**Photorespiration Does Not Contribute to Y(II) under CO$_2$-Limited Photosynthesis**

In general, the oxygenation of ribulose-1,5-bisphosphate by Rubisco is presumed to be suppressed in cyanobacteria, because CCM maintains [CO$_2$] high around Rubisco in the carboxysome (Badger and Price, 1992), but *Synechocystis* sp. PCC 6803 may be an exception (Eisenhut et al., 2006, 2008). Cyanobacterial photorespiration may operate through three routes, the canonical plant-like C2 cycle and two cyanobacteria-specific routes, the glycerate and decarboxylation pathways (Hagemann et al., 2013). Allahverdiyeva et al. (2011) demonstrated photorespiratory oxygen uptake in the flv1/3 double mutant by membrane inlet mass spectrometry. This opened the possibility that not only FLV2/4 but also photorespiration could be responsible for the increase in Y(II) under CO$_2$-limited photosynthesis in *Synechocystis* sp. PCC 6803 (Fig. 1). We constructed the mutants Δpgr, ΔglcD1/2, ΔgccT, and Δglyk, which were deficient in the genes encoding phosphoglycolate phosphatase (sll0458), glycolate dehydrogenase (sll0404 and sll0806), Gly decarboxylase T-protein (sll0171), and glyceraldehyde kinase (sll1840), respectively. These genes supposedly function in the canonical C2 cycle (Eisenhut et al., 2008). Inactivation of these genes in the mutants was confirmed by PCR analysis (Supplemental Figs. S2 and S3). All mutants showed the same Y(II) increase following the transition from CO$_2$-saturated to CO$_2$-limited photosynthesis as the wild-type *Synechocystis* sp. PCC 6803 (Supplemental Fig. S6; Supplemental Table S1), indicating that the C2 cycle was not involved in the Y(II) response.

**DISCUSSION**

We aimed at elucidating the molecular mechanism of AEF under CO$_2$-limited photosynthesis in *Synechocystis* sp. PCC 6803, reported in our previous study (Hayashi et al., 2014). On the transition from CO$_2$-saturated to CO$_2$-limited photosynthesis, AEF was transiently suppressed but then recovered to a value corresponding to the photosynthetic electron flux observed under CO$_2$-saturated photosynthesis. This implied the induction of AEF under CO$_2$-limited photosynthesis. We hypothesized that among the four FLV proteins in *Synechocystis* sp. PCC 6803, FLV2 and FLV4 could be involved because, first, the flv1/3 double mutant showed the same response as the wild type (Hayashi et al., 2014), and second, FLV2 and FLV4 were produced under low-[CO$_2$] conditions (Zhang et al., 2009; Eisenhut et al., 2012). We confirmed that
Synechocystis sp. PCC 6803 mutants deficient in FLV2 and FLV4 cannot maintain LEF during the transition to CO2-limited photosynthesis (Fig. 2), implying that FLV2 and FLV4 function in the AEF under CO2-limited photosynthesis. In summary (Fig. 4), we propose that under CO2-saturated photosynthesis, FLV1 and FLV3 operate (Helman et al., 2003; Allahverdiyeva et al., 2013; Hayashi et al., 2014) while FLV2 and FLV4 do not, whereas under CO2-limited photosynthesis, FLV2 and FLV4 operate in addition to FLV1 and FLV3 to mediate AEF, thus contributing to the alleviation of photooxidative stress (Zhang et al., 2009, 2012).

The most striking finding is that FLV2 and FLV4 together have the capacity to replace the steady-state photosynthetic electron flux observed under CO2-saturated photosynthesis (Figs. 1 and 2) by an AEF under CO2-limited conditions. In wild-type Synechocystis sp. PCC 6803 performing CO2-limited photosynthesis, Y(II) remained at 80% to 90% of that at CO2-saturated photosynthesis (Fig. 1). In Δflv2 and Δflv4 under CO2-limited conditions, Y(II) was only 5% to 10% of that at CO2-saturated photosynthesis, corresponding to the extent of the concomitant reduction of photosynthetic oxygen evolution (Fig. 2; Table I). These findings suggest that FLV2 and FLV4 are sufficient to maintain AEF activity under low [CO2].

Under CO2-depleted conditions, photorespiratory oxygen uptake in Δflv1/3 occurred at rates (about 50 μmol oxygen mg−1 Chl h−1) that exceeded the oxygen-photoreducing rate of FLV1/3 (approximately 30 μmol oxygen mg−1 Chl h−1; Allahverdiyeva et al., 2011), indicating that photorespiration can function as an electron sink under CO2-depleted as well as FLV1/3-depleted conditions (Helman et al., 2003; Allahverdiyeva et al., 2011). Unfortunately, we were unable to obtain evidence

Figure 3. Oxygen-reducing activities of recombinant GST and GST-FLV4 fusion protein. The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 1 mM NADH, and the protein of interest (40 μg). Catalase (Cat; 200 units mL−1) and dithionite (Dt; 1 mM) were added as indicated. Purified GST protein served as a control.

Figure 4. Model of the responses of photosynthesis, FLV activity, electron transport rate (ETR), and oxidation state of the PQ pool to the shift from CO2-saturated to CO2-limited photosynthesis in Synechocystis sp. PCC 6803. This model presents an interpretation of the processes occurring in the experiment shown in Figure 1A. A, CO2 depletion by photosynthesis. Medium [CO2] decreases due to photosynthetic consumption following the start of illumination. B, Suppression of photosynthesis by photosynthesis-induced CO2 depletion. Photosynthetic CO2 consumption cannot be balanced by the diffusion of atmospheric CO2 into the medium. C, Activities of FLV proteins. FLV1 and FLV3 function under CO2-saturated and CO2-limited conditions, while FLV2 and FLV4 become functional as mediators of AEF only under CO2-limited conditions. D, Total ETR under CO2-saturated photosynthesis is driven mainly by photosynthesis. A similar rate is maintained under CO2 limitation by an oxygen-dependent AEF. E, Changes in the redox state of the PQ pool. Under CO2-limited photosynthesis, the oxidized state is preserved by the FLV2/4-mediated, oxygen-dependent AEF.
in support of this idea (Supplemental Fig. S6). On the other hand, our data are in agreement with the interpretation that photorespiration operates in wild-type cells. For example, \( \Delta \text{gld} \text{D}1/2 \) and \( \Delta \text{gc} \text{v} \text{T} \) mutants showed retarded growth (data not shown), as reported by Eisenhut et al. (2006, 2008). In addition, the initial \( F_{\text{m}}' \) was lower in \( \Delta \text{gld} \text{D}1/2 \) than in other strains (Supplemental Fig. S6), possibly owing to phycobilisome functions including state transition. A physiological role for cyanobacterial photorespiration has been proposed by Eisenhut et al. (2006, 2008), who showed that in \textit{Synechocystis} sp. PCC 6803, photorespiration is not an electron sink but rather a scavenging system for toxic intermediates, notably glyoxylate (Eisenhut et al., 2008).

Supposedly, the electron acceptor for FLV2 and FLV4 is oxygen. Hayashi et al. (2014) reported that AEF activity under CO\(_2\)-limited photosynthesis in \textit{Synechocystis} sp. PCC 6803 cells requires oxygen. We showed that AEF requires FLV2 and FLV4 (Fig. 2) and that the GST-FLV4 fusion protein possesses oxygen-reducing activity (Fig. 3), similar to recombinant FLV3 (Vicente et al., 2002). These results suggest that FLV2 and FLV4 use oxygen as an electron acceptor in vivo and in vitro. In contrast, FLV2 and FLV4 interact with PSI and phycobilisomes (Zhang et al., 2012; Bersanini et al., 2014). Therefore, FLV2 and FLV4 may catalyze the photoreduction of oxygen to water associated with PSI under CO\(_2\)-limited conditions in \textit{Synechocystis} sp. PCC 6803. We did not succeed in preparing stable FLV2/4 heterodimers (data not shown). The molecular mechanism of FLV2/4 heterodimer function at PSI awaits its biochemical characterization in the future.

Under low-[CO\(_2\)] conditions, \( \Delta \text{fl} \text{e} \text{o} \text{2} \) and \( \Delta \text{fl} \text{v} \text{4} \) showed severe photoinhibition of PSI (Zhang et al., 2009, 2012). Moreover, an overexpression mutant of the \textit{fl}eo-2 operon in \textit{Synechocystis} sp. PCC 6803 exhibited a more oxidized state of the PQ pool and a reduced production of singlet oxygen and showed resistance to photoinhibition of PSI (Bersanini et al., 2014). In wild-type \textit{Synechocystis} sp. PCC 6803 under CO\(_2\)-limited photosynthesis, Y(II) remained high (Figs. 1 and 2; Hayashi et al., 2014), a property expected to contribute to the survival of \textit{Synechocystis} sp. PCC 6803 in nature, where CO\(_2\) assimilation would proceed under the CO\(_2\)-limited photosynthesis condition.

We attribute the low capacity for AEF in \textit{S. elongatus} PCC 7942 to the loss of FLV2 and FLV4. \textit{S. elongatus} PCC 7942 and \textit{Synechocystis} sp. PCC 6803 are \( \beta \)-type cyanobacteria, but in contrast to \textit{Synechocystis} sp. PCC 6803, \textit{S. elongatus} PCC 7942 does not harbor the gene for the orange carotenoid protein responsible for the nonphotochemical quenching of Chl fluorescence (Boulay et al., 2008). \textit{Synechocystis} sp. PCC 6803 mutants deficient in orange carotenoid protein suffer from photoinhibition under low-[CO\(_2\)] and high-light stress conditions (Wilson et al., 2006). The \textit{Synechocystis} sp. PCC 6803 mutant deficient in FLV2/4 showed photoinhibition and growth inhibition under these conditions (Zhang et al., 2009, 2012). This suggests that \textit{S. elongatus} PCC 7942 should lack tolerance against electron sink limitations such as those occurring under high light and low [CO\(_2\)] in contrast to \textit{Synechocystis} sp. PCC 6803. However, Bersanini et al. (2014) proposed that in cyanobacteria such as \textit{S. elongatus} PCC 7942 that lack FLV2 and FLV4, photoinhibition is alleviated by the expression of an additional copy of the reaction center protein in PSII, D1, called D1:2, which enhances the tolerance against photo stress (Clarke et al., 1993).

The relationship between this photoprotective system and FLV2/4 calls for further study.

Since the report of Asada and Badger (1984), the oxygen-dependent AEF (Mehler reaction in chloroplasts) has been quantitatively investigated in various photosynthetic organisms. In intact chloroplasts of higher plants, oxygen-dependent AEF amounted to 10% to 20% of the photosynthetic electron flux coupled to CO\(_2\) fixation (Badger et al., 2000). The proportion is as low as 1% to 10% according to a recent study, which would seem to suggest that the Mehler reaction is not a major electron sink in higher plants (Driever and Baker, 2011). In intact leaves of \( \text{C}_3 \) plants, photorespiration could be a major electron sink under conditions of CO\(_2\)-limited photosynthesis (Driever and Baker, 2011; Shirao et al., 2013). These conclusions cannot necessarily be extended to cyanobacteria and algae, which seem to have higher Mehler reaction activities than higher plants (Badger et al., 2000). In fact, the diatom \textit{Thalassiosira pseudonana} showed oxygen photoreduction at a rate of 600 \( \mu \)mol mg\(^{-1}\) Chl h\(^{-1}\), corresponding to 49% of the electron flux through PSII (Waring et al., 2010). Similarly, the oxygen-dependent electron transport rate contributes 30% of the total electron flux in \textit{Symbiodinium sp.}, a group of symbiotic dinoflagellates of cnidarians, and FLV proteins might be involved (Roberty et al., 2014). In view of these reports and this study, algae and cyanobacteria appear to utilize oxygen-dependent AEFs other than photorespiration as major alternative electron sinks. Linking the physiological function of oxygen-dependent AEFs with their diversity among photosynthetic organisms is a fascinating topic for future investigations.

**MATERIALS AND METHODS**

**Growth Conditions and Determination of Chl \( \alpha \)**

\textit{Synechocystis} sp. PCC 6803 and \textit{Synechococcus elongatus} PCC 7942 cells were cultured in BG-11 medium on a rotary shaker (100 rpm) under light/dark conditions (25°C, 16 h, 100 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\), fluorescent lamp/23°C, 8 h, dark) in 2% (v/v) CO\(_2\). For Chl quantification, cells were harvested and resuspended in 1 mL of 100% (w/v) methanol. After incubation at room temperature for 5 min, the suspension was centrifuged at 10,000g for 5 min at room temperature. Chl \( \alpha \) was determined by the method of Lee et al. (1998).

**Generation of Mutants**

To disrupt a gene (e.g., \textit{slr}0458; \textit{glp}D1, \textit{slr}0404; \textit{gxl}D2, \textit{slr}0806; \textit{gcv}T, \textit{slr}1017; \textit{gpl}, \textit{slr}1840; \textit{flv}2, \textit{slr}0019; and \textit{flv}4, \textit{slr}0021), the coding region was replaced with a cassette of the kanamycin resistance gene (\textit{Kan}) or the chloramphenicol resistance gene (\textit{Cmr}) amplified from \textit{pUC4-KKX} or \textit{pACYC184} vector, respectively (Nakahara et al., 2003), by PCR using appropriate \( f \) and \( r \) primer sets (listed in Supplemental Table S2). We constructed these mutants following
the method of Sakurai et al. (2007). The regions upstream and downstream of each gene were amplified by PCR using two sets of primers: up f and up r, and dn f and dn r, respectively. The front ends of up r and dn f contained regions that complemented the sequences of the primers Kan f and Kan r or Cm f and Cm r (Supplemental Table S2). We used three fragments, up, Kan or Cm, and dn, which were then linked by successive PCRs to obtain a disruption cassette. Sato et al. (2005) generated 40 mutants of *Synechocystis* sp. PCC 6803 by this high-throughput method.

Transformation of *Synechocystis* sp. PCC 6803 was performed by the standard procedure (Williams, 1988). Transformants were selected on 0.5% (w/v) agar plates of BG-11 medium containing 20 μg mL⁻¹ kanamycin or 30 μg mL⁻¹ chloramphenicol. The double mutant ΔgcrD1Δ2 was generated by transformation of the ΔgcrD2 (gcrD868) mutant with the mutated ΔgcrD1 (gcrD864). The resultant transformant was selected on 0.5% (w/v) agar plates of BG-11 medium containing 20 μg mL⁻¹ kanamycin and 30 μg mL⁻¹ chloramphenicol.

Measurement of Oxygen Concentration

The uptake and evolution of oxygen were measured simultaneously with Chl fluorescence. The reaction mixture (2 mL) containing 50 mM HEPES-KOH (pH 7.5) and cyanobacterial cells (10 μg Chl mL⁻¹) was illuminated with actinic light (red light, greater than 620 nm; 200 μmol photons m⁻² s⁻¹) at 25°C. During the measurements, the reaction mixture was stirred with a magnetic stirrer. In one set of experiments, oxygen concentrations were monitored continuously with an oxygen electrode while the measuring cuvette remained open to allow the diffusion of oxygen and CO₂ between the medium and the atmosphere (Hansatech). Hayashi et al., 2014. Typical results of this type of experiment are shown in Figures 1 and 2. In a second set of experiments, the top of the cuvette was temporarily (1–3 min) closed to determine the oxygen evolution rate. Typical data for the determination of oxygen evolution rate are shown in Supplemental Figure S1.

Measurement of Chl Fluorescence

The relative Chl fluorescence originating from Chl a was measured using a PAM-Chl fluorometer (PAM-101; Walz; Hayashi et al., 2014). Pulse-modulated excitation was achieved by a light-emitting diode lamp with peak emission at 680 nm. Modulated fluorescence was measured at the wavelength (>710 nm; Schott RC9 long-pass filter). The minimum Chl fluorescence was determined by illumination with measuring light. The F₁ was measured under actinic red light, and 1,000-ms pulses of saturated light (10,000 μmol photons m⁻² s⁻¹) were applied at arbitrary intervals to determine the F₄o. Y(II) was defined as (F₄o − F₁)/F₄o. The fluorescence terminology used in this study follows van Kooten and Bjelk (1990).

Cloning and Expression of Recombinant GST-FLV2/4 Fusion Proteins

The coding regions of FLV2 and FLV4 were obtained from the genomic DNA of *Synechocystis* sp. PCC 6803 using KOD-EX Neo (Toyobo) and subcloned via BamHI into pGEX-4T-3 (GE Healthcare) using the In-Fusion HD cloning kit (Takara). The primers for these genes are listed in Supplemental Table S3. Recombinant proteins were produced in BL21 (Agilent Technology) host cells. Overnight cultures of the transformed BL21 cells in Luria-Bertani broth were used to inoculate (0.1% v/v) fresh Luria-Bertani broth, which was then incubated at 37°C until the A₆₀₀ reached 0.5 to 1. Protein expression was evaluated at 15°C for 16 h in Luria-Bertani broth containing 0.1 mM isopropyl β-D-thiogalactopyranoside.

Purification of Recombinant GST Fusion Proteins

Cells were harvested by centrifugation and resuspended in phosphate-buffered saline buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, and 1 mM phenylmethylsulfonyl fluoride [pH 7.3]). After gentle sonication, the resulting crude extract was centrifuged at 15,000 rpm for 30 min at 4°C. The lysate was loaded onto GSTrap FF columns (GE Healthcare). Unbound proteins were removed by washing with phosphate-buffered saline buffer. Recombinant GST fusion proteins were then eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, and 1 mM phenylmethylsulfonyl fluoride [pH 8]). The purified recombinant protein was quantified using the Pierce 660-nm protein assay (Thermo Scientific). Antibodies against the N-terminal of the recombinant proteins were produced using SDS-PAGE and stained with Coomassie Brilliant Blue to evaluate the purity of the recombinant proteins (Supplemental Fig. S4).

Enzyme Assays

The NADH-oxidizing activity of recombinant GST-FLV4 fusion protein was evaluated in a reaction mixture (1 mL) containing 50 mM Tris-HCl (pH 7.4), 10 μg of GST-FLV4 protein, and various concentrations of NADH. The FLV-dependent oxidation of NADH was monitored as the decrease in A₅₅₀ at 25°C, assuming an absorption coefficient of 6.22 cm⁻¹. The oxygen-reducing activities of the recombinant GST and GST-FLV4 proteins were evaluated in a reaction mixture (2 mL) containing 50 mM Tris-HCl (pH 7.4), 1 mM NADH, and the protein of interest (40 μg). GST protein was used as the control. GST- and GST-FLV-dependent oxygen reductions were measured with an oxygen electrode (Hansatech).

Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Determination of oxygen evolution rates during the development of photosynthetic parameters in *Synechocystis* sp. PCC 6803 wild type.

**Supplemental Figure S2.** Maps of the insertion sites of antibiotic resistance cassettes in ΔgcrD1, ΔgcrD2, ΔgcvT, ΔΔgxyk, ΔDio2, and ΔDio4.

**Supplemental Figure S3.** DNA fragments amplified by PCR showing complete segregation of the inactivated genes *pgp*, *gcrD1Δ2*, *gcvT*, ΔΔgxyk, and ΔDio4.

**Supplemental Figure S4.** SDS-PAGE and western blot of GST, GST-FLV2, and GST-FLV4 proteins.

**Supplemental Figure S5.** Development of the NADH oxidation activity on the concentration of NADH in the reaction catalyzed by the recombinant GST-FLV4 protein.

**Supplemental Figure S6.** Development of photosynthetic parameters in media containing high-[CO₂]-grown Δpgp, ΔgcrD1Δ2, ΔgcvT, and ΔΔgxyk mutants of *Synechocystis* sp. PCC 6803.

**Supplemental Table S1.** Oxygen-exchange rates in the dark, under CO₂-saturated and CO₂-limited photosynthesis, and after the addition of NaHCO₃ in four mutants of *Synechocystis* sp. PCC 6803 (Δpgp, ΔgcrD1Δ2, ΔgcvT, and ΔΔgxyk).

**Supplemental Table S2.** Oligonucleotides used for the disruption of the gene *gcv*.

**Supplemental Table S3.** Oligonucleotides used for the construction of FLV2 and FLV4 expression vectors.

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Table S1. O₂ exchange rates in the dark, under CO₂-saturated and CO₂-limited photosynthesis, and after addition of NaHCO₃, in four mutants of S. 6803 (∆pgp, ∆glcD1/2, ∆gcvT, and ∆glyk).

|                  | ∆pgp | ∆glcD1/2 | ∆gcvT | ∆glyk |
|------------------|------|----------|-------|-------|
| Dark             | −47 ± 19 | −20 ± 6 | −22 ± 12 | −13 ± 4 |
| CO₂-saturated photosynthesis (α) | 110 ± 10 | 110 ± 17 | 122 ± 3 | 125 ± 16 |
| CO₂-limited photosynthesis (β) | 8.5 ± 2.3 | 5.9 ± 1.7 | 12 ± 7 | 8 ± 4 |
| Addition of NaHCO₃ (γ) | 98 ± 23 | 94 ± 8 | 98 ± 9 | 115 ± 7 |

O₂ exchange rates were determined simultaneously with Chl fluorescence measurement under actinic light (AL; 200 μmol photons m⁻² s⁻¹) at the times indicated by α, β, and γ in Supplemental Fig. S6 (see also Fig. 1 and 2). The reaction mixture contained cyanobacterial cells (10 μg Chl mL⁻¹) and 50 mM HEPES-KOH (pH 7.5). The O₂ exchange rates in the dark were determined before illumination with measuring light (ML). Negative values indicate O₂ uptake. Values (μmol O₂ [mg Chl]⁻¹ h⁻¹) are means ± standard deviation, n = 4.

Table S1. Shimakawa et al. (2014)
Table S2. Oligonucleotides used for the disruption of the \textit{pgp}, \textit{glcD1/2}, \textit{gcvT}, \textit{glyk}, \textit{flv2}, and \textit{flv4} genes.

| Primer name | Sequence (5'-3') | Primer name | Sequence (5'-3') |
|-------------|------------------|-------------|------------------|
| \textit{Kan} \textit{f}  | TAGACTGGGGCGGTTTTATGG | \textit{sll0171} up \textit{f}  | ATTCTCTGAGCGCGAGAAATAGG |
| \textit{Kan} \textit{r}  | ATTCCGAAGCCCAACCTTT | \textit{sll0171} up \textit{r}  | CCATAAAAACCCCGAGTCGATACAACTGAATGCTGAAAA |
| \textit{Cmr} \textit{f}  | GATCGGCACGTAAGAGGTTTC | \textit{sll0171} \textit{dn} \textit{f}  | AAAGGTGGGGCTTCGAGATCGGAAAGAACAACCTCTGG |
| \textit{Cmr} \textit{r}  | AGAAGGCACAGGTGACTAGT  | \textit{sll0171} \textit{dn} \textit{r}  | CCCCTAAATCCACTAATAAAG |
| \textit{slr0458} \textit{up} \textit{f}  | GGAACCTACATTCGAGCCCTTAGC | \textit{slr1840} up \textit{f}  | TTTGATTGCACGAGACTCTTTT |
| \textit{slr0458} \textit{up} \textit{r}  | CCATAAAAACCCCGAGTCGATACAACTGAATGCTGAAAA | \textit{slr1840} up \textit{r}  | CCATAAAAACCCCGAGTCGATACAACTGAATGCTGAAAA |
| \textit{slr0458} \textit{dn} \textit{f}  | AAAGGTGGGGCTTCGAGATCGGAAAGAACAACCTCTGG |
| \textit{slr0458} \textit{dn} \textit{r}  | GGGAGACTGATGCAAAAACTCAC | \textit{slr1840} \textit{dn} \textit{r}  | TGTTCACCTGAACAAATCTTTT |
| \textit{slr0404} \textit{up} \textit{f}  | GTGCTACCTGTTCCGTTG | \textit{slr0219} up \textit{f}  | CATTCTGCTCATTGCTGGTGT |
| \textit{slr0404} \textit{up} \textit{r}  | GAACTCTTACGTGGCGATCGAAGATCTAATCCACAGCAAA | \textit{slr0219} up \textit{r}  | CCATAAAAACCCCGAGTCGATACAACTGAATGCTGAAAA |
| \textit{slr0404} \textit{dn} \textit{f}  | CAGTGTGACCGTGTTCTTCTATGCTAATCTTGGCTGCTCA | \textit{slr0219} \textit{dn} \textit{f}  | AAAGGTGGGGCTTCGAGATCGGAAAGAACAACCTCTGG |
| \textit{slr0404} \textit{dn} \textit{r}  | TGCCCAATATGTTCAACGAA | \textit{slr0219} \textit{dn} \textit{r}  | ACAGAAACACGGTTTGCTCAGT |
| \textit{slr0806} \textit{up} \textit{f}  | CTTGGTTTTGCACCAAAAAAT | \textit{slr0217} up \textit{f}  | GCCATAGACCCCTCCTGAGAT |
| \textit{slr0806} \textit{up} \textit{r}  | CCATAAAAACCCCGAGTCGATACAACTGAATGCTGAAAA | \textit{slr0217} up \textit{r}  | GAACCTCTTACGTGGCGATCGAAGATCTAATCTTGGCTGCTCA |
| \textit{slr0806} \textit{dn} \textit{f}  | AAAGGTGGGGCTTCGAGATCGGAAAGAACAACCT | \textit{slr0217} \textit{dn} \textit{f}  | CAGTGTGACCGTGTTCTTCTATGCTAATCTTGGCTGCTCA |
| \textit{slr0806} \textit{dn} \textit{r}  | AATACACCTCGGGCAAACCTG | \textit{slr0217} \textit{dn} \textit{r}  | GAATCCACATTGGGTAGGGAAG |

All sequences are displayed in the 5′-to-3′ orientation.

Table S2. Shimakawa et al. (2014)
Table S3. Oligonucleotides used for the construction of FLV2 and 4 expression vectors. All sequences are displayed in the 5′-to-3′ orientation.

| Primer name     | Sequence (5′-3′)                                      |
|-----------------|------------------------------------------------------|
| FLV2-Infusion f | GGTTCCGCGTGGATCCATGATTTCTCCCAATTGGTGTTTCT           |
| FLV2-Infusion r | GGGAATTCCGGATCTCAATAGTTGGTCGCCCG                     |
| FLV4-Infusion f | GGTTCCGCGTGGATCCATGTTACCCTTCAATTGATTCT              |
| FLV4-Infusion r | GGGAAATTCCGGGGATCTTAGTAGTGGTTGCCCAGTTT              |

All sequences are displayed in 5′-3′ orientation.
Fig. S1. Determination of O₂ evolution rates during the monitoring of medium [O₂] (red trace) and relative Chl fluorescence (black trace) in the S. 6803 wild type. A representative experiment is shown. At times α, β, and γ, the top of the O₂ electrode chamber was closed for 1–3 min (indicated by blue shading), and the O₂ evolution rates were determined. Thereafter, the chamber was opened again. For the measurement of the rate at β, the sensitivity of the recording was increased temporarily from 2 V to 500 mV. The medium containing S. 6803 cells (10 µg Chl mL⁻¹) was illuminated with actinic light (200 µmol photons m⁻² s⁻¹) from the indicated starting time (AL). The cells were added to the medium before illumination with measuring light commenced (ML). Further details as in Fig. 1.
Fig. S2. Physical maps of the insertion sites of antibiotic resistance cassettes in Δpgp, ΔglcD1/2, ΔgcvT, Δglyk, Δflv2, and Δflv4. The arrowheads indicate the locations of PCR primers (Supplemental Table S2). The predicted lengths of the amplified DNA fragments are shown. The full lengths of the pgp, glcD1, glcD2, gcvT, glyk, flv2, and flv4 genes are 636 bp, 1,479 bp, 1,320 bp, 1,119 bp, 1,115 bp, 1,785 bp, and 1,737 bp, respectively. The shaded boxes indicate the removed regions (Δpgp, 54 bp; ΔglcD1, 133 bp; ΔglcD2, 966 bp; ΔgcvT, 116 bp; Δglyk, 235 bp; Δflv2, 689 bp; Δflv4, 430 bp).
Fig. S3. DNA fragments amplified by PCR showing complete segregation of the inactivated genes, pgp (slr0458), glcD1 (sll0404), glcD2 (slr0806), gcvT (sll0171), glyk (slr1840), flv2 (sll0219), and flv4 (sll0217).
**Fig. S4.** SDS-PAGE and Western blot of GST, GST-FLV2, and GST-FLV4 proteins. Purified recombinant proteins (0.1 μg each) were analyzed by SDS–PAGE; the gel was stained with CBB. GST, GST-FLV2, and GST-FLV4 proteins were detected by the GST-antibody in Western-bLOTS. Expected sizes of recombinant proteins are 26 kDa (GST), 92 (= 26 + 66) kDa (GST-FLV2), and 90 (= 26 + 64) kDa (GST-FLV4).
Fig. S5. Dependence of the NADH-oxidation activity on the concentration of NADH in the reaction catalyzed by the recombinant GST-FLV4 protein.
Fig. S6. Development of photosynthetic parameters in media containing four high-[CO₂]-grown deletion mutants of *S*. 6803, Δ*pgp* (top left), Δ*glcD1/2* (bottom left), Δ*gcvt* (top right), and Δ*glyk* (bottom right). Other details as in Fig. 1.