



# History of the Fight of Oxygenic Phototrophs against Reactive Oxygen Species~Establishing the novel concept “P700 oxidation system” ~

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(Degree)

博士（農学）

(Date of Degree)

2018-03-25

(Date of Publication)

2019-03-01

(Resource Type)

doctoral thesis

(Report Number)

甲第7212号

(URL)

<https://hdl.handle.net/20.500.14094/D1007212>

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# Doctoral Dissertation

## History of the Fight of Oxygenic Phototrophs against Reactive Oxygen Species

~Establishing the novel concept “P700 oxidation system”~

光合成生物が活性酸素に抗ってきた歴史

～新規コンセプト「P700 酸化システム」の確立に至るまで～

January 2018

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## 1. Comprehensive Summary

In this doctoral thesis, I aim to document our research that established the novel concept “P700 oxidation system” and uncovered the physiological role of oxygen ( $O_2$ ) therein in oxygenic phototrophs as described in the latest Japanese review (嶋川銀河 and 三宅親弘, 2017). The research was performed in a laboratory in Kobe University with the supports by a number of colleagues based on Chikahiro Miyake’s original research proposals.

Oxygenic photosynthesis is an essential biological reaction for living things on the earth, because it produces organic compounds from carbon dioxide ( $CO_2$ ) with water ( $H_2O$ ) and photon energy, releasing  $O_2$  as the byproduct. It seems to be a severe challenge that 3 billion years ago the progenitor of oxygenic phototrophs (i.e., cyanobacteria) had begun photosynthesis on the earth, because  $O_2$  evolved as the byproduct of photosynthesis has the potential to generate reactive oxygen species (ROS) to cause oxidative damage in the cells. At present, oxygenic phototrophs have thrived on the earth in a variety forms, including cyanobacteria, algae, and plants, although  $O_2$  occupies 21% of the atmosphere. Overall, the 3 billion years has been the history of the fights of oxygenic phototrophs against the potential risk of photosynthesis (i.e., ROS).

The goal of our research is to uncover the physiological strategies and molecular mechanisms to prevent the production of ROS in oxygenic phototrophs. Recently, our laboratory has demonstrated that oxygenic phototrophs alleviate the ROS production by maintaining the reaction center chlorophyll (Chl) in photosystem I (PSI), P700, in its oxidized form. The oxidation of P700 is regulated by various molecular mechanisms, altogether termed as “P700 oxidation system”. In the Part I section in this doctoral thesis, we showed diverse responses of photosynthesis to  $CO_2$  limited conditions, where the risk of the ROS production is elevated, in cyanobacteria and green algae. We additionally discussed the diversity of the

photosynthetic responses to CO<sub>2</sub> limitation in the secondary algae, including diatoms and euglenoids, in Part II. Thereafter, we showed in Part III the diverse strategy to oxidize P700 under CO<sub>2</sub> limitation among cyanobacteria. That is, P700 oxidation is conserved as the universal strategy to alleviate the production of ROS in oxygenic phototrophs. Nevertheless, cyanobacteria, algae, and plants have developed diverse molecular mechanisms and show various photosynthetic responses to the situations where ROS can be generated. Evolutionary processes should have affected the diverse strategies to oxidize P700. Therefore, in Part IV we investigated the P700 oxidation system in the liverwort *Marchantia polymorpha*, which is the evolutionary bridge between algae and plants. From these reports, we discussed the history that oxygenic phototrophs have gone through for 3 billion years facing ROS.

## 2. Abbreviations

AEF, alternative electron flow

AL, actinic light

ARTO, cytochrome *b<sub>o</sub>*-type quinol oxidase

ATPase, chloroplast ATP synthase

CCM, CO<sub>2</sub> concentrating mechanism

CEF, cyclic electron flow

Chl, chlorophyll

COX, *aa<sup>3</sup>*-type cytochrome *c* oxidase

Cyd, cytochrome *bd*-type quinol oxidase

Cyt *b<sub>6</sub>/f*, cytochrome *b<sub>6</sub>/f* complex

DIRK, dark-interval relaxation kinetics

ECS, electrochromic shift

ETR, electron transport rate

Fd, ferredoxin

FLV, flavodiiron protein

F<sub>m</sub>' , maximum variable fluorescence under saturating light

F<sub>o</sub>, minimum fluorescence determined under measuring light

F<sub>s</sub>, steady-state fluorescence under actinic light

F<sub>v</sub>/F<sub>m</sub>, maximum quantum efficiency of PSII photochemistry

gH<sup>+</sup>, proton conductance of the chloroplast ATP synthase

HA, hydroxylamine

LEF, photosynthetic linear electron flow

ML, measuring light

NDH, chloroplast NADPH dehydrogenase

NPQ, non-photochemical quenching

OCP, orange carotenoid protein

P680, the reaction center chlorophyll in photosystem II

P680<sup>+</sup>, photo-oxidized P680

P700, the reaction center chlorophyll in photosystem I

P700<sup>+</sup>, photo-oxidized P700

PAM, pulse-amplitude modification

PFD, photon flux density

PGR5, proton gradient regulation 5

P<sub>m</sub>, total photo-oxidizable P700

PQ, plastoquinone

PQH<sub>2</sub>, plastoquinol

PSI, photosystem I

PSII, photosystem II

PTOX, plastidial terminal oxidase

qP, photochemical quenching of chlorophyll fluorescence based on puddle model

R<sub>d</sub>, dark respiration rate

RISE, reduction-induced suppression of electron flow

ROS, reactive oxygen species

rSP illumination, repetitive short-pulse illumination

RTO, cyanobacterial respiratory terminal oxidase

Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBP, ribulose-1,5-bisphosphate

S. 6803, *Synechocystis* sp. PCC 6803

S. 7002, *Synechococcus* sp. PCC 7002

S. 7942, *Synechococcus elongatus* PCC 6803

SP, short-saturation pulse

WT, wild type

Y(I), effective quantum yield of photosystem I

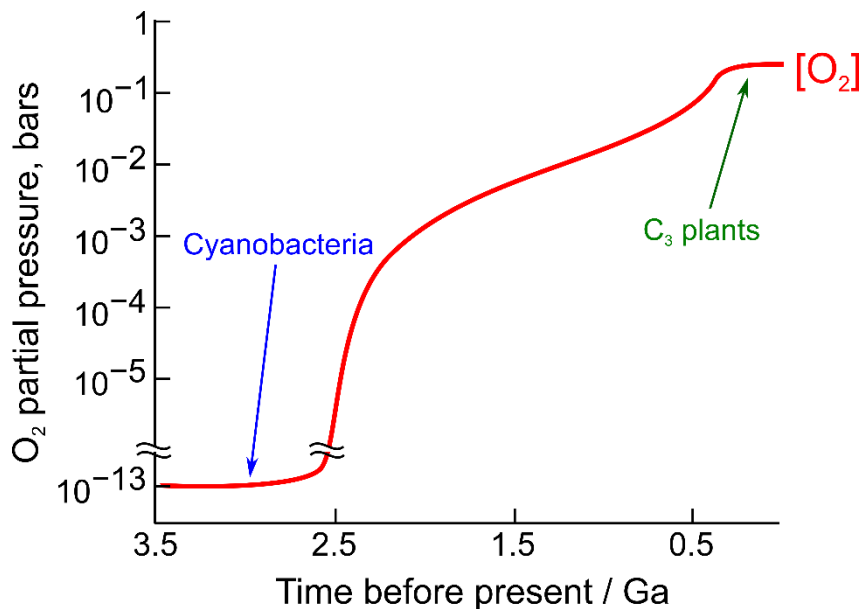
Y(II), effective quantum yield of photosystem II

$\Delta\text{pH}$ , a proton gradient across the thylakoid membrane

$\Delta\psi$ , a membrane potential across the thylakoid membrane

### 3. Comprehensive Introduction

Air consists of 21% O<sub>2</sub>, the concentration of which increased during the evolution of oxygenic phototrophs, in particular the oceanic cyanobacteria, around 2–3 billion years ago (Fig. 1) (Kasting, 1987). Due to its electron configuration, O<sub>2</sub> has a very high oxidizing potential and is the final electron acceptor in aerobic respiratory electron transport. Additionally, O<sub>2</sub> can be converted to ROS, including superoxide anion radical (O<sub>2</sub><sup>•−</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (•OH), and singlet oxygen (<sup>1</sup>O<sub>2</sub>) (Asada, 2006).



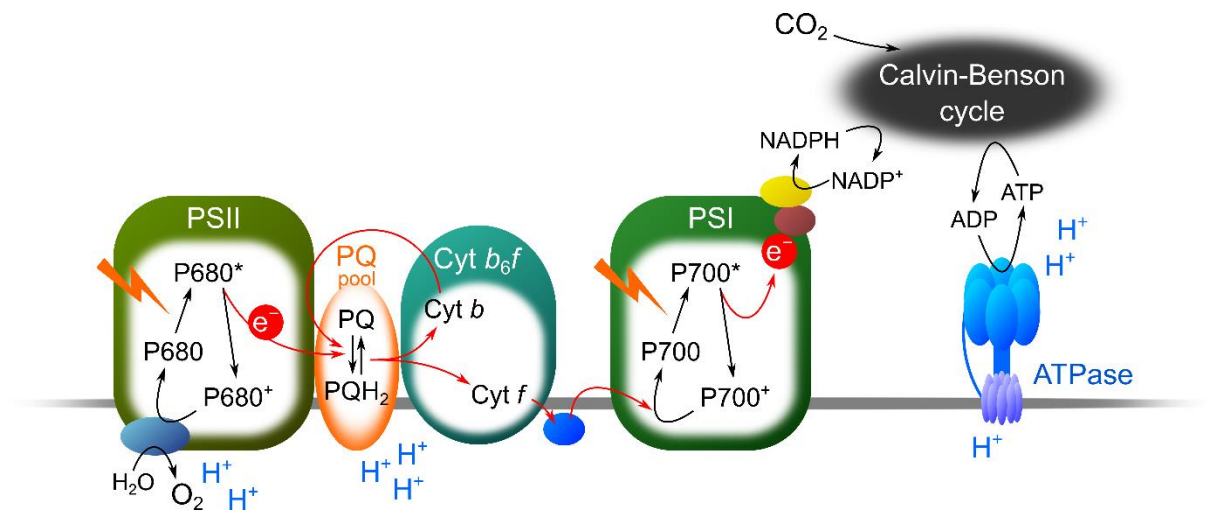
Modified from Kasting (1987)

**Fig. 1.** Change of the partial pressure of O<sub>2</sub>. The data are shown in reference to the previous report (Kasting, 1987).

Oxygenic phototrophs are vulnerable to photo-oxidative damage by ROS that originate from the photosynthetic electron transport system (Jones and Kok, 1966; Satoh, 1970). During

the light reaction of photosynthesis, photon energy is absorbed by the chlorophyll in photosystem (PS) I and II on the thylakoid membrane to stimulate the photo-oxidation/reduction cycles of the Chl reaction centers P680 (at PSII) and P700 (at PSI), thereby driving photosynthetic linear electron flow (LEF) from PSII to PSI through the plastoquinone (PQ) pool, the cytochrome *b<sub>6</sub>/f* complex (Cyt *b<sub>6</sub>/f*), and plastocyanin (or cytochrome *c<sub>6</sub>*) (Fig. 2) (Govindjee, 2012). On the electron acceptor side of PSI, NADP<sup>+</sup> is reduced to NADPH with electrons from PSI *via* ferredoxin (Fd) and Fd-NADP<sup>+</sup> reductase. Conversely, in PSII, H<sub>2</sub>O is oxidized to O<sub>2</sub> on the luminal side of the thylakoid membrane as the oxidized P680 (P680<sup>+</sup>) accepts electrons from H<sub>2</sub>O *via* a Mn complex (Najafpour and Sedigh, 2013). In Cyt *b<sub>6</sub>/f*, the Q-cycle pumps stromal H<sup>+</sup> to the luminal side (Tikhonov, 2014). These processes establish a proton gradient ( $\Delta pH$ ) across the thylakoid membrane to produce ATP *via* chloroplast ATP synthase (ATPase). The resulting NADPH and ATP are utilized for CO<sub>2</sub> assimilation in the Calvin–Benson cycle (Lichtenthaler et al., 2015). Limitation during photosynthetic CO<sub>2</sub> assimilation under low CO<sub>2</sub> and high light intensity can cause accumulation of excess electrons in the photosynthetic electron transport system. Sejima et al. (2014) simulated such conditions by applying repetitive short-pulse illumination (e.g., 20,000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 300 ms, every 10 s) and found that PSI was rapidly inactivated (i.e. PSI photoinhibition) *via* ROS (Terashima et al., 1994; Sonoike, 2011; Cazzaniga et al., 2012; Sejima et al., 2014; Zivcak et al., 2015; Zivcak et al., 2015; Takagi et al., 2016). The repair of inactivated PSI is a very slow process (Kudoh and Sonoike, 2002), and a recent study showed that it took more than 12 days for damaged PSI in wheat leaves to recover completely (Zivcak et al., 2015). Therefore, PSI photoinhibition is very detrimental to oxygenic phototroph growth.

Usually, PSI photoinhibition is rarely observed, and oxygenic phototrophs can tolerate sunlight (2000–3000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) that far exceeds the demands of photosynthesis. Recently, Sejima et al. (2014) confirmed that ROS production is suppressed when P700 is



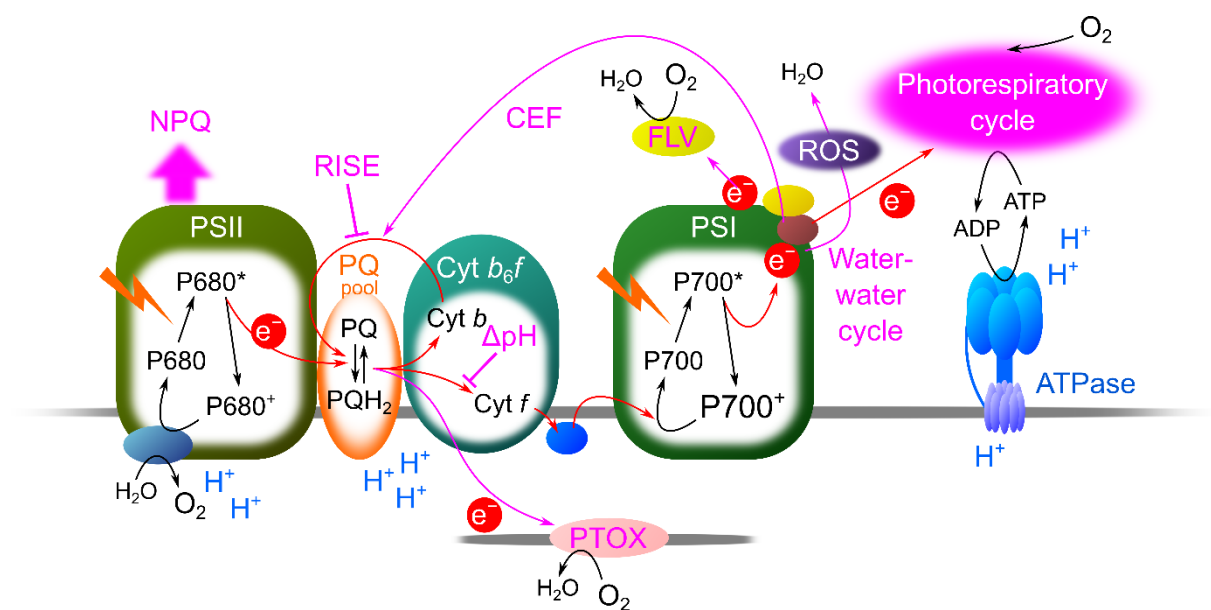
**Fig. 2.** An overall picture of LEF in oxygenic phototrophs. Red lines indicate the electron transport pathways.

maintained in its oxidized form ( $P700^+$ ), because of the decrease of P700 in the ground state, which is the source of both excess electrons and energy to produce ROS. The oxidation of P700 is recognised as a universal physiological response to excessive light conditions, such as high light and low  $CO_2$  (Badger and Schreiber, 1993; Klughammer and Schreiber, 1994; Golding and Johnson, 2003; Miyake et al., 2005). These facts indicate that P700 oxidation is the strategy that prevents photo-oxidative damage caused by ROS in PSI.

The oxidation of P700 is expected to be controlled by some regulatory mechanisms on both the electron acceptor and donor sides of PSI (Fig. 3). On the acceptor side, photosynthetic  $CO_2$  assimilation and  $O_2$ -dependent alternative electron flow (AEF) function as electron sinks, thereby probably contributing to P700 oxidation. In oxygenic phototrophs, various  $O_2$ -dependent AEF have been reported, including photorespiration (Farquhar et al., 1982; Ogren, 1984; Kozaki and Takeba, 1996; Wiese et al., 1998; Driever and Baker, 2011), water-water

cycle (or Mehler reaction) (Mehler, 1951; Schreiber and Neubauer, 1990; Miyake et al., 1991; Miyake and Asada, 1992), Mehler-like reaction mediated by flavodiiron protein (FLV) (Helman et al., 2003; Helman et al., 2005; Zhang et al., 2009; Allahverdiyeva et al., 2011; Zhang et al., 2012; Allahverdiyeva et al., 2013; Hasunuma et al., 2014; Hayashi et al., 2014; Shimakawa et al., 2015), and chlororespiration catalysed by plastidial terminal oxidase (PTOX) or cyanobacterial respiratory terminal oxidase (RTO) (Nixon, 2000; Carol and Kuntz, 2001; Pils and Schmetterer, 2001; Joët et al., 2002; Lea-Smith et al., 2013). On the donor side of PSI, the suppression of electron transport into PSI is supposed to cause P700 oxidation. Basically, LEF is limited at the step of the oxidation of reduced PQ (i.e. plastoquinol, PQH<sub>2</sub>) in Cyt *b<sub>6</sub>/f* (Stiehl and Witt, 1969; Anderson, 1992; Kirchhoff et al., 2000; Yamori et al., 2011; Schöttler et al., 2015). However, in response to short-term environmental fluctuations, the electron transport in Cyt *b<sub>6</sub>/f* is modulated by a regulatory mechanism, which is believed to be strongly associated with  $\Delta$ pH across the thylakoid membrane. The acidification on the luminal side of the thylakoid membrane limits the electron transport in Cyt *b<sub>6</sub>/f* (Nishio and Whitmarsh, 1993) and induces the dissipation of excess photon energy as heat at PSII (i.e. qE quenching) reflected as non-photochemical quenching (NPQ) of chlorophyll fluorescence (Ruban et al., 2012; Derks et al., 2015; Ruban, 2016). The formation of  $\Delta$ pH is coupled with total electron transport activity in the photosynthetic electron transport (Kanazawa and Kramer, 2002) and is adjusted by the proton conductance (gH<sup>+</sup>) of ATPase (Takizawa et al., 2008; Rott et al., 2011) or other ion transporters (Armbruster et al., 2014). Furthermore,  $\Delta$ pH formation is possibly promoted by cyclic electron flow (CEF) around PSI (Allen, 2003; Nandha et al., 2007). Conversely, the suppression of electron transport into PSI for P700 oxidation (i.e. reduction-induced suppression of electron transport, RISE) has been observed in a cyanobacteria species (Shaku et al., 2016). In RISE, the suppression of electron transport in Cyt *b<sub>6</sub>/f* is triggered by the reduction of the PQ pool, but not the accumulation of  $\Delta$ pH, which implies that RISE is caused

by the suppression of the Q-cycle in Cyt  $b_6/f$  because of a shortage of oxidized PQ (Shaku et al., 2016). However, no evidence suggests that RISE functions in photosynthetic eukaryotes. For the last two decades, P700 oxidation has been observed as a universal physiological response to excess light conditions. Nevertheless, the molecular mechanisms underlying P700 oxidation in oxygenic phototrophs are still poorly understood.



**Fig. 3.** An overall picture of hypothetical P700 oxidation system. The candidates of the molecular mechanisms for P700 oxidation are shown as pink. Red and pink arrows indicate the electron transport pathways.

## Part I

# Diversity in photosynthetic electron transport under [CO<sub>2</sub>]-limitation in the cyanobacterium *Synechococcus* sp. PCC 7002 and green alga *Chlamydomonas reinhardtii*

This part is based on the following publication:

**Ginga Shimakawa, Seiji Akimoto, Yoshifumi Ueno, Ayumi Wada, Keiichiro Shaku, Yuichiro Takahashi, Chikahiro Miyake** (2016) Diversity in photosynthetic electron transport under [CO<sub>2</sub>]-limitation: the cyanobacterium *Synechococcus* sp. PCC 7002 and green alga *Chlamydomonas reinhardtii* drive an O<sub>2</sub>-dependent alternative electron flow and non-photochemical quenching of chlorophyll fluorescence during CO<sub>2</sub>-limited photosynthesis. *Photosynthesis Research* 130(1) 293–305.

#### 4.1. Abstract

Some cyanobacteria, but not all, experience an induction of AEF during CO<sub>2</sub>-limited photosynthesis. For example, *Synechocystis* sp. PCC 6803 (S. 6803) exhibits AEF, but *Synechococcus elongatus* PCC 7942 (S. 7942) does not. This difference is due to the presence of FLV 2 and 4 proteins, i.e., FLV2/4, in S. 6803, which catalyze electron donation to O<sub>2</sub>. In this study, we observed a low-[CO<sub>2</sub>] induced AEF in the marine cyanobacterium *Synechococcus* sp. PCC 7002 (S. 7002) that lacks FLV2/4. The AEF shows high affinity for O<sub>2</sub>, compared with AEF mediated by FLV2/4 in S. 6803, and can proceed under extreme low [O<sub>2</sub>] (about a few μM O<sub>2</sub>). Further, the transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited photosynthesis leads a preferentially excitation of PSI to PSII and increased non-photochemical quenching of chlorophyll fluorescence. We found that the model green alga *Chlamydomonas reinhardtii* also has an O<sub>2</sub>-dependent AEF showing the same affinity for O<sub>2</sub> as that in S. 7002. These data represent the diverse molecular mechanisms to drive AEF in cyanobacteria and green algae. In this paper, we further discuss the diversity, the evolution, and the physiological function of strategy to CO<sub>2</sub>-limitation in cyanobacterial and green algal photosynthesis.

## 4.2. Introduction

Under low [CO<sub>2</sub>] or high-light conditions, the regeneration of NADP<sup>+</sup>, an electron acceptor for PSI, limits the electron transport rate in the photosynthetic electron transport system, unless an AEF can substitute its rate (Hayashi et al., 2014; Shimakawa et al., 2015). For example, the low [CO<sub>2</sub>]-inducible AEF in the cyanobacterium S. 6803 is activated within an hour after the cells are exposed to low [CO<sub>2</sub>] (Hayashi et al., 2014). The induced AEF requires O<sub>2</sub>, and its magnitude corresponds to that of electron flux in the photosynthetic electron transport system for photosynthesis (Hayashi et al., 2014). As a consequence, the oxidation state of photosynthetic electron transport system is maintained even under the limitation of NADP<sup>+</sup> regeneration in S. 6803. On the other hand, the cyanobacterium S. 7942 does not induce AEF upon exposure to low [CO<sub>2</sub>], causing the photosynthetic electron transport system to be reduced under CO<sub>2</sub>-limitation (Miller et al., 1996; Hayashi et al., 2014). Overall, the induction of AEF depends on the species of cyanobacteria. FLV2/4 have been identified to catalyze the O<sub>2</sub>-dependent AEF, which functions under low [CO<sub>2</sub>] in S. 6803 (Shimakawa et al., 2015), as FLV1/3 do in the photosynthesis induction and under fluctuating light (Helman et al., 2003; Helman et al., 2005; Allahverdiyeva et al., 2011; Allahverdiyeva et al., 2013). The genes encoding FLV2/4 in S. 6803 are expressed within an hour after exposure to low [CO<sub>2</sub>] conditions (Zhang et al., 2009; Zhang et al., 2012). In contrast, S. 7942 does not have the genes for FLV2/4 (Fujisawa et al., 2014; Fujisawa et al., 2017). Furthermore, FLV2/4-knockout mutants of S. 6803 do not show AEF under low [CO<sub>2</sub>], as in S. 7942 (Hayashi et al., 2014; Shimakawa et al., 2015), which indicates that there are no other systems than FLV2/4 to be an alternate for the electron flux in photosynthesis in both S. 6803 and S. 7942, under low [CO<sub>2</sub>].

There are several molecular mechanisms of AEF in oxygenic phototrophs besides FLVs. First, Mehler reaction occurs in the acceptor side of PSI, where O<sub>2</sub> is reduced to O<sub>2</sub><sup>-</sup>

ultimately converted to H<sub>2</sub>O by superoxide dismutase and ascorbate peroxidase (termed as the water-water cycle) (Mehler, 1951; Miyake and Asada, 1992, 2003). Second, respiratory terminal oxidases such as cytochrome *c* oxidase, *bd*-type quinol oxidase, alternative respiratory oxidase (altogether termed as cyanobacterial RTOs), and PTOX also mediate O<sub>2</sub>-dependent AEF (Beardall et al., 2003; Peltier et al., 2010; Lea-Smith et al., 2013). Third, the oxygenation reaction of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) drives photorespiration, which consumes excess photon energy (Kozaki and Takeba, 1996; Sejima et al., 2016). Finally, CEF is also known as an AEF, which donates electrons from the acceptor side of photosystems to the donor side of them around PSI and PSII (Arnon et al., 1954; Falkowski et al., 1986; Miyake and Yokota, 2001; Miyake et al., 2002; Shikanai, 2014, 2016).

In cyanobacteria, two molecular mechanisms different from AEF that suppress the electron inputs from PSII to the photosynthetic electron transport system have been proposed to alleviate the oxidative damage from ROS. These mechanisms are observed as NPQ of Chl fluorescence (Demmig-Adams et al., 2012; Ruban, 2016). In *S. 6803*, soluble orange carotenoid protein (OCP) is expressed at high rates after cells are transferred to high-light conditions (Hihara et al., 2001), which contributes to the NPQ induced by strong white or blue-green light (Wilson et al., 2006; Sedoud et al., 2014). In addition, the *S. 6803* mutant deficient in OCP suffers from photoinhibition of PSII (Wilson et al., 2006; Kusama et al., 2015). Interestingly, the gene responsible for OCP appears to occur only in some species of cyanobacteria, since it has been observed in *S. 6803*, but not in *S. 7942*. Overall, the molecular mechanisms to alleviate the oxidative damage are not the same in all cyanobacteria species, suggesting that its evolutionary development may have originated in the cyanobacterial line. Nonetheless, state transitions are observed in almost all cyanobacteria and related to NPQ (Murata, 1969, 1969; van Thor et al., 1998; Papageorgiou et al., 2007). In state I, there is more antenna in PSII, whereas in state II there is more antenna in PSI. A state I to state II transition is characterized

by a decrease in maximum Chl fluorescence yield of PSII, and it probably reduces oxidative damage (McConnell et al., 2002; Fujimori et al., 2005). On the other hand, state II to state I is characterized by increased fluorescence for the simple reason that the yield of PSII fluorescence is much higher than that of PSI (Papageorgiou and Govindjee, 2004).

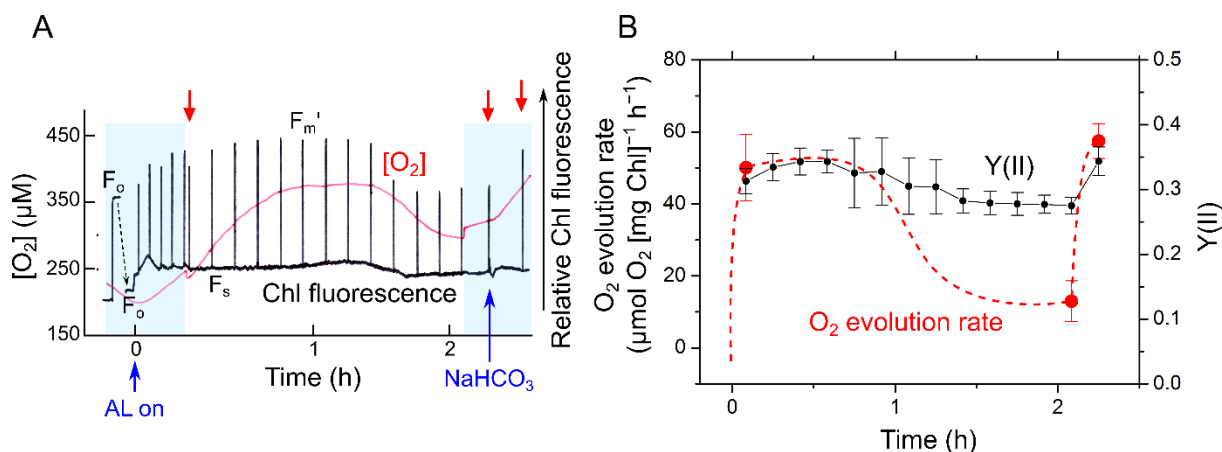
In this study, we focused on the marine cyanobacterium S. 7002. In the phylogenetic tree based on rRNA and whole genome among cyanobacteria, the evolutionary path for S. 7002 is similar to that of S. 6803 (Turner et al., 1999; Satoh et al., 2013). However, different from S. 6803, S. 7002 does not have genes encoding for FLV2/4 (Fujisawa et al., 2014; Fujisawa et al., 2017). In addition, the S. 7002 lineage is different from other *Synechococcus* species such as S. 7942 (Dvořák et al., 2014). We investigated the response of electron flux in the photosynthetic electron transport system to CO<sub>2</sub>-limited conditions in S. 7002. Similar to S. 6803, S. 7002 grown under ambient [CO<sub>2</sub>] displayed an induction of AEF after exposure to CO<sub>2</sub>-limitation, maintaining the photosynthetic electron transport system electron flux. On the other hand, the cells grown under high [CO<sub>2</sub>] did not show the AEF, similar to S. 7942. Overall, AEF is required for growth under suppressed photosynthetic conditions. Additionally, the AEF observed in S. 7002 has high affinity for O<sub>2</sub>, compared with that in S. 6803. Furthermore, the model green alga *Chlamydomonas reinhardtii* (hereafter *C. reinhardtii*) also showed an O<sub>2</sub>-dependent AEF, similar to S. 7002. From these results, we characterize this AEF process and discuss the physiological functions and evolutionary processes in S. 7002 and *C. reinhardtii*.

### 4.3. Results

#### **Transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited photosynthesis in ambient [CO<sub>2</sub>] and high [CO<sub>2</sub>] grown S. 7002**

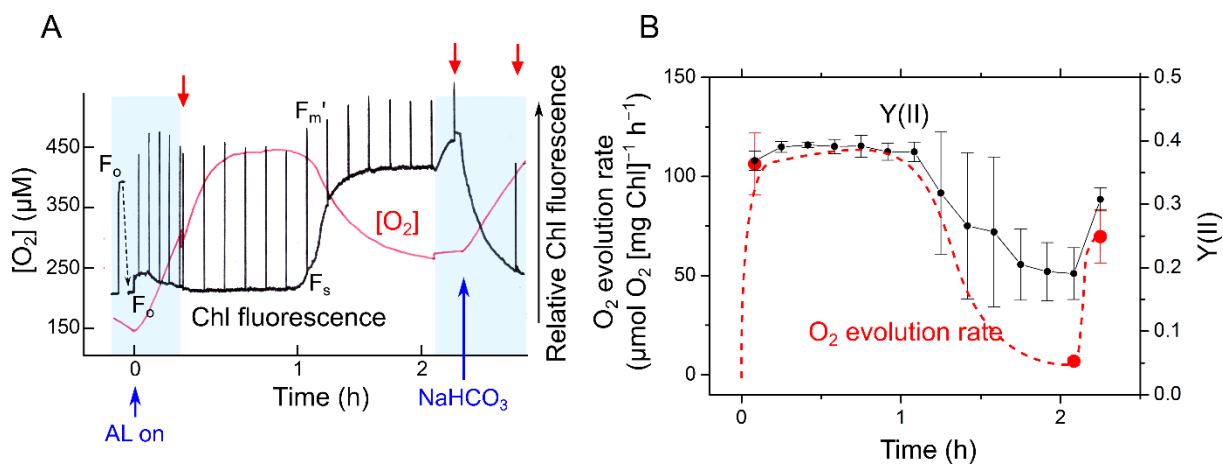
We simultaneously monitored relative Chl fluorescence and [O<sub>2</sub>] in fresh A<sup>+</sup> medium that contained S. 7002 cells (10 µg Chl mL<sup>-1</sup>) without NaHCO<sub>3</sub> addition, by the method of Shimakawa et al. (2015). Before the cells were added to the medium, we checked the ambient [O<sub>2</sub>] (about 250 µM at 25 °C). First, we measured dark respiration rates of the cells grown under ambient [CO<sub>2</sub>]. Next, we turned on the red AL (620 nm <  $\lambda$  < 695 nm, 150 µmol photons m<sup>-2</sup> s<sup>-1</sup>) to monitor the induction phase of photosynthesis. Upon illumination, [O<sub>2</sub>] in the medium increased and the relative Chl fluorescence increased from F<sub>0</sub> to F<sub>s</sub>, which subsequently decreased transiently, indicating the start of photosynthesis. The O<sub>2</sub> evolution rates obtained after the photosynthesis induction at  $\alpha$  were defined as the photosynthesis rates when enough CO<sub>2</sub> exists in the medium (hereafter “the CO<sub>2</sub>-saturated photosynthesis”) (Shimakawa et al., 2015). After the O<sub>2</sub> evolution rates at  $\alpha$  were determined, we opened the top of the reaction chamber to allow equilibration of the medium with air. We thereafter monitored the relative Chl fluorescence and [O<sub>2</sub>] with the intervals of saturation pulses illumination changed (from 1 min to 10 min; Fig. 4A). After a steady-state photosynthesis, [O<sub>2</sub>] decreased continuously, which was paralleled by a decreasing F<sub>m</sub>' while the F<sub>s</sub> did not change. These suggests a suppression of photosynthesis and the occurrence of NPQ in this phase (at  $\beta$ ; Fig. 4A). Addition of CO<sub>2</sub> (as 10 mM NaHCO<sub>3</sub>) caused an increase in [O<sub>2</sub>] and Y(II), and a relaxation of NPQ (at  $\gamma$ ; Fig. 4A), indicating that the lower O<sub>2</sub> evolution rates and Y(II) at  $\beta$  were due to a shortage of available CO<sub>2</sub> (Shimakawa et al., 2015). To obtain the O<sub>2</sub> evolution rate at  $\beta$  and  $\gamma$ , we temporarily closed the top of the reaction chamber. We defined the O<sub>2</sub> evolution rates at  $\beta$  and  $\gamma$  (Fig. 4B)

respectively as the photosynthesis rates when the medium's  $\text{CO}_2$  is the results of photosynthetic  $\text{CO}_2$  consumption and diffusion from the air (hereafter “the  $\text{CO}_2$ -limited photosynthesis”), and the  $\text{CO}_2$ -saturated photosynthesis respectively (high  $\text{CO}_2$  is available due to the addition of  $\text{NaHCO}_3$ ) (Shimakawa et al., 2015).



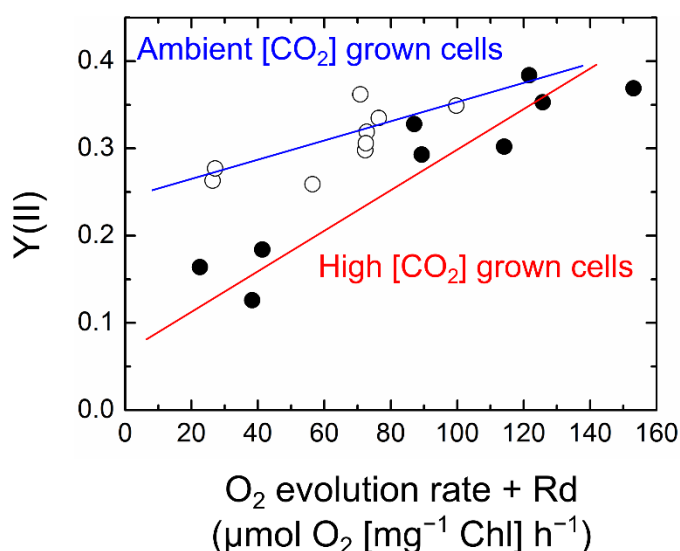
**Fig. 4.** Development of photosynthetic parameters in media containing ambient  $[\text{CO}_2]$  grown *S. 7002*. (A) Medium  $\text{O}_2$  concentration (red) and relative Chl fluorescence (black) through time ( $n = 3$ ). Measurements on medium with cyanobacteria ( $10 \mu\text{g Chl mL}^{-1}$ ) started in dark conditions; illumination with measuring light (ML) and red AL ( $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) started as indicated. The downward shift in the Chl fluorescence signal ( $F_o$ ) after the start of ML illumination is due to the increase in instrument amplification from 200 to 100 mV. Chl fluorescence parameters have the usual definitions ( $F_o$ , minimum fluorescence determined under ML;  $F_s$ , steady-state fluorescence under AL;  $F_m'$ , maximum variable fluorescence under saturating light).  $\text{NaHCO}_3$  (final concentration 10 mM) was added as indicated. Red arrows indicate the times at which  $\text{O}_2$  evolution rates were determined. Blue shadings indicate the oxygen electrode chamber was closed, the  $[\text{O}_2]$  signal amplitude was doubled, and measurement time scales (x-axis) were reduced to 1/5. (B) Time courses of net  $\text{O}_2$  evolution rate and  $Y(\text{II})$ , calculated as  $(F_m' - F_s)/F_m'$ ; means  $\pm$  SD ( $n = 3$ ).

Next, we measured the *S. 7002* cells grown under high  $[\text{CO}_2]$ . Interestingly, in contrast to the ambient  $[\text{CO}_2]$  grown cells, the  $F_s$  increased paralleled by a decreasing  $\text{O}_2$  evolution rate (Fig. 5A), indicating that the PQ pool in the photosynthetic electron transport system is reduced under  $\text{CO}_2$ -limitation. As a result, the decrease in  $Y(\text{II})$  in the transition from  $\text{CO}_2$ -saturated to  $\text{CO}_2$ -limited photosynthesis was larger in high  $[\text{CO}_2]$  grown cells than the cells grown under ambient  $[\text{CO}_2]$  (Fig. 4B and 5B). That is, there is little AEF to replace photosynthesis during  $\text{CO}_2$ -limited photosynthesis in high  $[\text{CO}_2]$  grown *S. 7002*. The  $\text{CO}_2$ -limited photosynthesis in high  $[\text{CO}_2]$  grown *S. 7002* was similar to that of *S. 7942*, because the *flv4-2* operon is lacking (Hayashi et al., 2014; Shimakawa et al., 2015; 嶋川銀河, 2015).



**Fig. 5.** Development of photosynthetic parameters in media containing high  $[\text{CO}_2]$  grown *S. 7002*. (A) Medium  $\text{O}_2$  concentration (red) and relative Chl fluorescence (black) through time ( $n = 3$ ). Measurements on medium with cyanobacteria ( $10 \mu\text{g Chl mL}^{-1}$ ) started in dark conditions; illumination with ML and red AL ( $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) started as indicated. The downward shift in  $F_0$  after the start of ML illumination is due to the increase in instrument amplification from 200 to 100 mV. NaHCO<sub>3</sub> (final concentration 10 mM) was added as

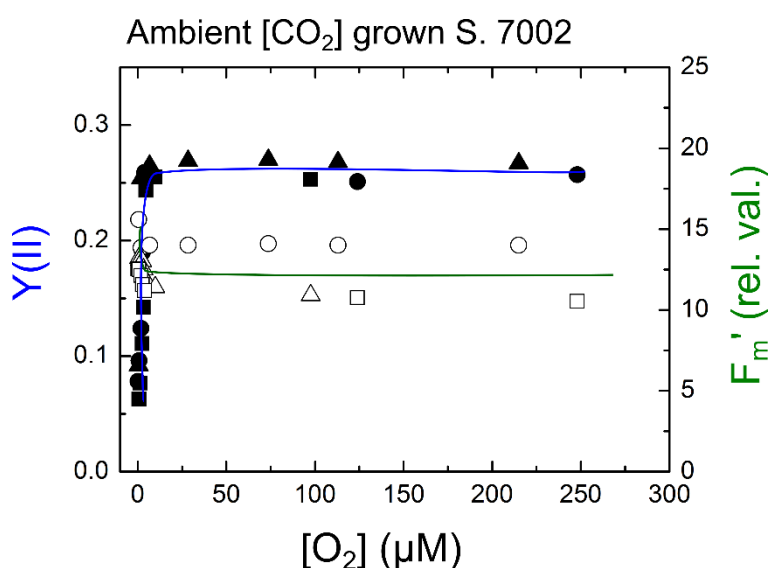
indicated. Red arrows indicate the times at which  $O_2$  evolution rates were determined. Blue shadings indicate the oxygen electrode chamber was closed, the  $[O_2]$  signal amplitude was doubled, and measurement time scales (x-axis) were reduced to 1/5. (B) Time courses of net  $O_2$  evolution rate and  $Y(II)$ , calculated as  $(F_m' - F_s)/F_m'$ ; means  $\pm$  SD ( $n = 3$ ).



**Fig. 6.** The relationship between photosynthetic  $O_2$  evolution rate and  $Y(II)$  in ambient  $[CO_2]$  grown (open circle) and high  $[CO_2]$  grown (closed circle) *S. 7002*.

We compared the  $O_2$  evolution rates and  $Y(II)$  at  $\alpha$ ,  $\beta$ , and  $\gamma$ , of ambient  $[CO_2]$  grown *S. 7002* with those in high  $[CO_2]$  grown cells at the same time (Fig. 6). On the basis of the proportional relationship between photosynthetic  $O_2$  evolution and electron transport through PSII (Campbell et al., 1998),  $Y(II)$  should decrease with a decrease in the  $O_2$  evolution rate in both ambient and high  $[CO_2]$  grown *S. 7002*. However, for the cells grown under ambient  $[CO_2]$ , the decrease in  $Y(II)$  was small, compared with that for the cells grown under high  $[CO_2]$ , and

it was estimated that about 80%  $Y(II)$  of that during  $CO_2$ -saturated photosynthesis was uncoupled with photosynthesis under  $CO_2$ -limitation (Fig. 6). These results indicate that the AEF is expressed particularly in ambient  $[CO_2]$  grown cells, and functions to replace photosynthesis during  $CO_2$ -limited photosynthesis though it may proceed to some extent also during  $CO_2$ -saturated photosynthesis.



**Fig. 7.** Dependence of  $Y(II)$  on  $[O_2]$  during  $CO_2$ -limited photosynthesis in ambient  $[CO_2]$  grown *S. 7002*. Closed symbols and open symbols represent  $Y(II)$  and  $F_m'$ , respectively. Measurements were performed three times indicated by circles, triangles, and squares respectively. For the measurements, *D*-glucose (5 mM), catalase (250 units  $mL^{-1}$ ), and glucose oxidase (5 units  $mL^{-1}$ ) were added to the media.

### The dependence of the AEF on $[O_2]$ in ambient $[CO_2]$ grown *S. 7002*

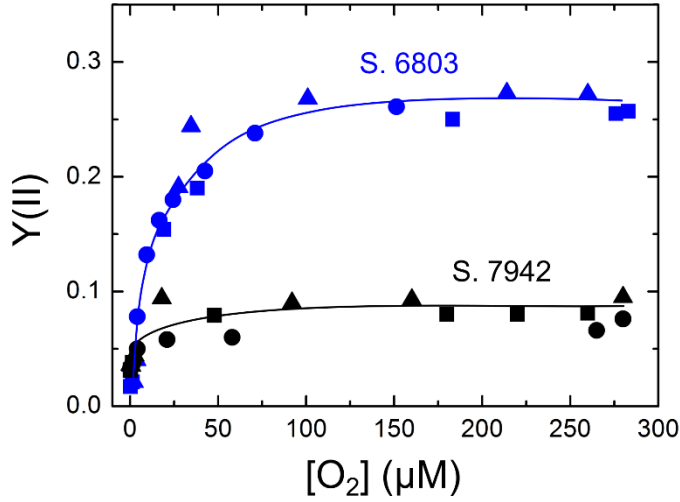
To examine the molecular mechanism of the AEF observed during  $CO_2$ -limited photosynthesis

in ambient [CO<sub>2</sub>] grown S. 7002, we investigated the dependence of Y(II) on [O<sub>2</sub>] under CO<sub>2</sub>-limitation as described below (Fig. 7). First, the cyanobacterial cells were analyzed to the measurement as described above. We added glucose and catalase (Wako, from bovine liver) to the medium during CO<sub>2</sub>-limited photosynthesis, and then closed and sealed the top of the chamber to block a diffusion of air to the medium. After the O<sub>2</sub> evolution rate decreased to zero, we added glucose oxidase (Wako, from *Aspergillus niger*) by a microsyringe and removed the dissolved O<sub>2</sub> in the medium. For the measurement, we confirmed no effect of adding glucose (for 10 min) on O<sub>2</sub> evolution rate and Y(II) during CO<sub>2</sub>-limited photosynthesis in S. 7002 in advance (Table 1). Surprisingly, the Y(II) did not change when [O<sub>2</sub>] was a few  $\mu$ M, different from S. 6803 (Fig. 8) (Hayashi et al., 2014), and decreased to about 20% of Y(II) under air-level [O<sub>2</sub>] when it was less than 1  $\mu$ M (Fig. 7), which indicates that the AEF during CO<sub>2</sub>-limited photosynthesis has high affinity for O<sub>2</sub> than the O<sub>2</sub>-dependent AEF mediated by FLV2/4 in S. 6803 (Hayashi et al., 2014; Shimakawa et al., 2015). On the other hand, the residual Y(II) contains a component of AEF that does not require O<sub>2</sub> (Fig. 7).

**Table 1.** Effects of glucose on O<sub>2</sub> evolution rates and Y(II) during CO<sub>2</sub>-limited photosynthesis in S. 7002, S. 6803, and S. 7942 grown under ambient [CO<sub>2</sub>] ( $n = 3$ )

| Strain  | O <sub>2</sub> evolution rate |             | Y(II)           |                 |
|---------|-------------------------------|-------------|-----------------|-----------------|
|         | Control                       | + D-Glucose | Control         | + D-Glucose     |
| S. 7002 | 13 $\pm$ 2                    | 13 $\pm$ 3  | 0.26 $\pm$ 0.01 | 0.27 $\pm$ 0.02 |
| S. 6803 | 3 $\pm$ 2                     | 4 $\pm$ 3   | 0.25 $\pm$ 0.02 | 0.25 $\pm$ 0.02 |
| S. 7942 | 4 $\pm$ 2                     | 4 $\pm$ 2   | 0.10 $\pm$ 0.01 | 0.11 $\pm$ 0.01 |

*Final concentration of D-glucose is 5 mM. Control shows the data before glucose is added.*

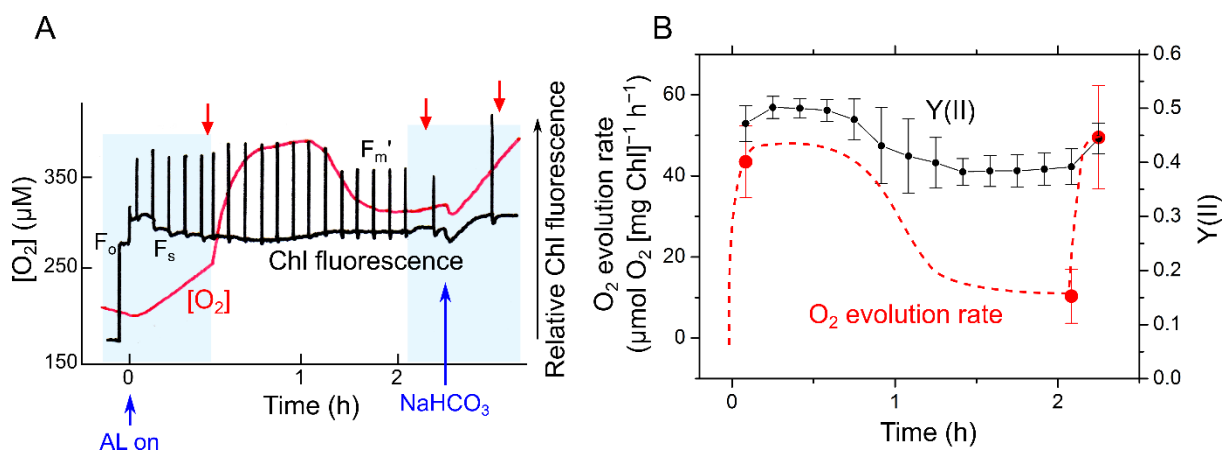


**Fig. 8.** Dependence of  $Y(II)$  on  $[O_2]$  during  $CO_2$ -limited photosynthesis in ambient  $[CO_2]$  grown *S. 6803* (blue) and *S. 7942* (black). Measurements were performed three times indicated by circles, triangles, and squares respectively. For the measurements, *D*-glucose (5 mM), catalase (250 units  $mL^{-1}$ ), and glucose oxidase (5 units  $mL^{-1}$ ) were added to the media.

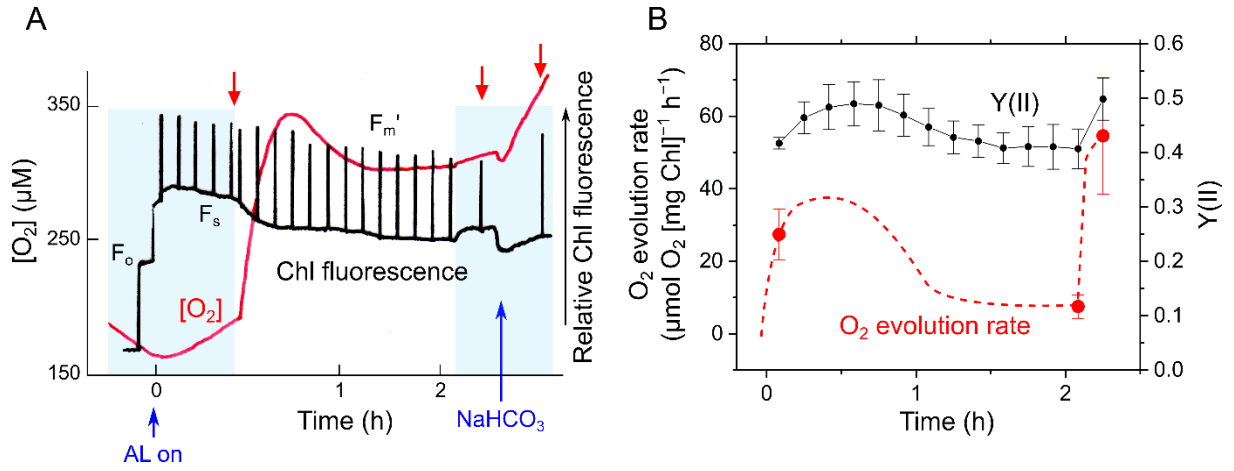
### Transition from $CO_2$ -saturated to $CO_2$ -limited photosynthesis in ambient $[CO_2]$ and high $[CO_2]$ grown *C. reinhardtii*

In this study, we measured  $CO_2$ -limited photosynthesis in the green alga *C. reinhardtii*. Eukaryotic green algae do not have the FLV2/4 encoding genes (Zhang et al., 2009). In *C. reinhardtii*, regardless of  $[CO_2]$  throughout the growth conditions,  $F_s$  did not change in the transition from  $CO_2$ -saturated to  $CO_2$ -limited photosynthesis, similar to ambient  $[CO_2]$  grown *S. 7002*, while  $F_m'$  decreased in the transition to  $CO_2$ -limited photosynthesis (Fig. 9 and 10). The decrease in  $F_m'$  was observed particularly in ambient  $[CO_2]$  grown cells (Fig. 9A and 10A), which suggests a larger NPQ in ambient  $[CO_2]$  grown cells than that in high  $[CO_2]$  grown cells.

Additionally, the decrease in  $Y(II)$  was larger in ambient  $[CO_2]$  grown cells in the transition from  $CO_2$ -saturated photosynthesis to  $CO_2$ -limited photosynthesis than that in high  $[CO_2]$  grown cells (Fig. 9B and 10B). From the relationships between photosynthetic  $O_2$  evolution rates and  $Y(II)$ , it was estimated that more than half of  $Y(II)$  during  $CO_2$ -saturated photosynthesis were kept where photosynthesis hardly proceed in *C. reinhardtii* (Fig. 11). These data indicate that an AEF replace photosynthesis during  $CO_2$ -limited photosynthesis in *C. reinhardtii*.



**Fig. 9.** Development of photosynthetic parameters in media containing ambient  $[CO_2]$  grown *C. reinhardtii*. (A) Medium  $O_2$  concentration (red) and relative Chl fluorescence (black) through time ( $n = 3$ ). Measurements on medium with algae ( $10 \mu g Chl mL^{-1}$ ) started in dark conditions; illumination with ML and red AL ( $200 \mu mol photons m^{-2} s^{-1}$ ) started as indicated.  $NaHCO_3$  (final concentration 10 mM) was added as indicated. Red arrows indicate the times at which  $O_2$  evolution rates were determined. Blue shadings indicate the oxygen electrode chamber was closed, and measurement time scales (x-axis) were reduced to 1/10. (B) Time courses of net  $O_2$  evolution rate and  $Y(II)$ , calculated as  $(F_m' - F_s)/F_m'$ ; means  $\pm SD$  ( $n = 3$ ).

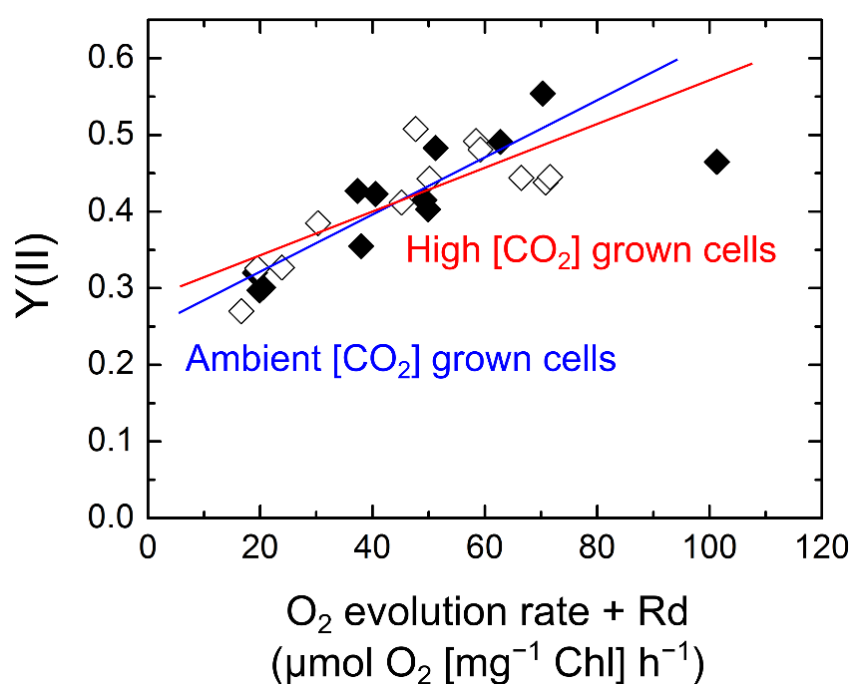


**Fig. 10.** Development of photosynthetic parameters in media containing high  $[CO_2]$  grown *C. reinhardtii*. (A) Medium  $O_2$  concentration (red) and relative Chl fluorescence (black) through time ( $n = 3$ ). Measurements on medium with algae ( $10 \mu g Chl mL^{-1}$ ) started in dark conditions; illumination with ML and red AL ( $200 \mu mol photons m^{-2} s^{-1}$ ) started as indicated.  $NaHCO_3$  (final concentration 10 mM) was added as indicated. Red arrows indicate the times at which  $O_2$  evolution rates were determined. Blue shadings indicate the oxygen electrode chamber was closed, and measurement time scales (x-axis) were reduced to 1/10. (B) Time courses of net  $O_2$  evolution rate and  $Y(II)$ , calculated as  $(F_m' - F_s)/F_m'$ ; means  $\pm SD$  ( $n = 3$ ).

### The dependence of the AEF on $[O_2]$ in *C. reinhardtii*

We investigated the dependence of  $Y(II)$  on  $[O_2]$  during  $CO_2$ -limited photosynthesis in *C. reinhardtii* by the same method in Fig. 7 and 8 to determine the requirement of  $O_2$  in the AEF under  $CO_2$ -limitation in *C. reinhardtii* (Fig. 12). For the measurement, we confirmed no effect of adding glucose on the  $O_2$  evolution rate and the  $Y(II)$  during  $CO_2$ -limited photosynthesis in *C. reinhardtii* in advance (Table 2). The AEF in *C. reinhardtii* partially showed dependence on

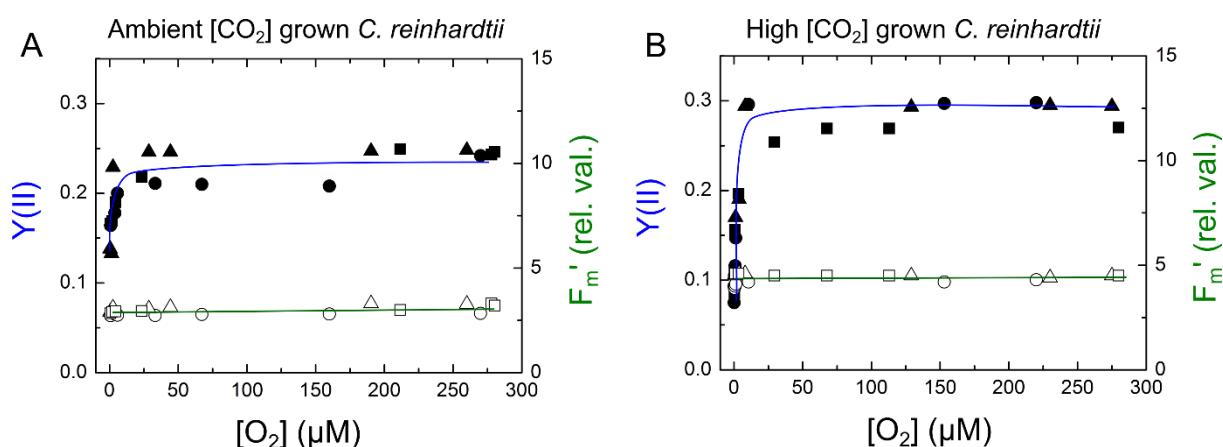
O<sub>2</sub>, but the dependencies differed between ambient and high [CO<sub>2</sub>] grown cells (Fig. 12). In ambient grown [CO<sub>2</sub>] cells, a half Y(II) uncoupled with photosynthesis was sensitive to extreme low [O<sub>2</sub>] while the other half does not depend on O<sub>2</sub> (Fig. 12A). On the other hand, most Y(II) was dependent on O<sub>2</sub> in the cells grown under high [CO<sub>2</sub>] (Fig. 12B). In sum, in *C. reinhardtii*, an O<sub>2</sub>-dependent AEF proceeds under CO<sub>2</sub>-limitation and has high affinity for O<sub>2</sub> as well as that in ambient [CO<sub>2</sub>] grown *S. 7002*.



**Fig. 11.** The relationship between photosynthetic O<sub>2</sub> evolution rate and Y(II) in ambient [CO<sub>2</sub>] grown (open diamond) and high [CO<sub>2</sub>] grown (closed diamond) *C. reinhardtii*.

**Change of the distribution of excitation energy in the transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited photosynthesis in *S. 7002* and *C. reinhardtii***

The distribution of excitation energy between two photosystems was monitored by measuring Chl fluorescence spectra at a low temperature (Ley and Butler, 1980), since  $F_m'$  decreased in the transition from  $\text{CO}_2$ -saturated to  $\text{CO}_2$ -limited photosynthesis in both S. 7002 and *C. reinhardtii*. Previously, it was found that state transition does not occur in the transition from  $\text{CO}_2$ -saturated to  $\text{CO}_2$ -limited photosynthesis in cyanobacteria, including S. 6803 and S. 7942 (Miller et al., 1996; Hayashi et al., 2014). However, in ambient  $[\text{CO}_2]$  grown S. 7002, the relative Chl fluorescence derived from PSII was lower during  $\text{CO}_2$ -limited photosynthesis, compared with that during  $\text{CO}_2$ -saturated photosynthesis (Fig. 13A). Additionally, the same change of excitation energy distribution was observed in ambient  $[\text{CO}_2]$  grown *C. reinhardtii* (Fig. 13B).



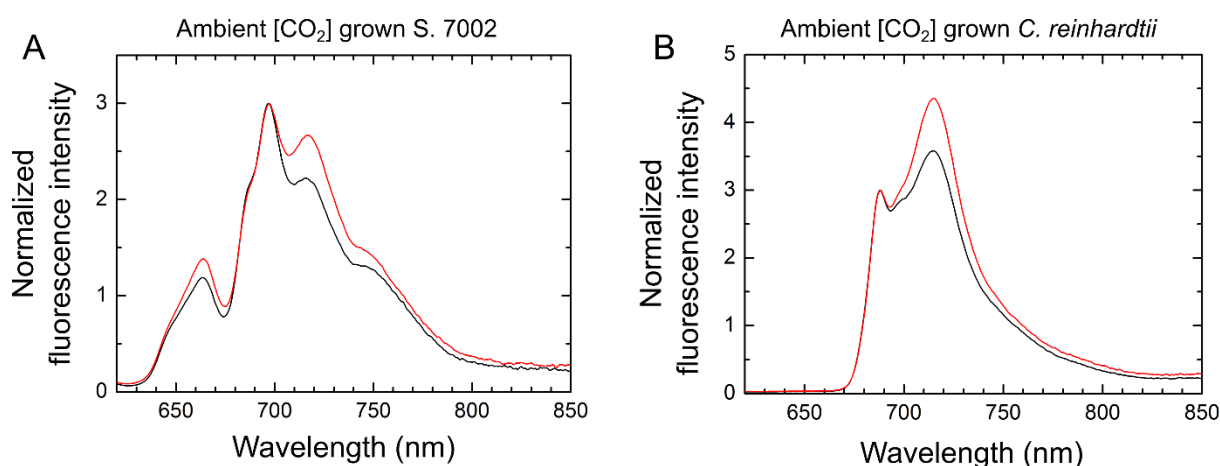
**Fig. 12.** Dependence of  $Y(II)$  on  $[\text{O}_2]$  during  $\text{CO}_2$ -limited photosynthesis in ambient  $[\text{CO}_2]$  (A) and high  $[\text{CO}_2]$  (B) grown *C. reinhardtii*. Closed symbols and open symbols represent  $Y(II)$  and  $F_m'$ , respectively. Measurements were performed three times indicated by circles, triangles, and squares respectively. For the measurements, D-glucose (5 mM), catalase (250 units  $\text{mL}^{-1}$ ), and glucose oxidase (5 units  $\text{mL}^{-1}$ ) were added to the media.

**Table 2.** Effects of glucose on O<sub>2</sub> evolution rates and Y(II) during CO<sub>2</sub>-limited photosynthesis in *C.*

*reinhardtii* grown under ambient and high [CO<sub>2</sub>] (*n* = 3)

| Strain                     | O <sub>2</sub> evolution rate |             | Y(II)       |             |
|----------------------------|-------------------------------|-------------|-------------|-------------|
|                            | Control                       | + D-Glucose | Control     | + D-Glucose |
| Ambient [CO <sub>2</sub> ] | 9 ± 5                         | 10 ± 5      | 0.34 ± 0.03 | 0.34 ± 0.03 |
| High [CO <sub>2</sub> ]    | 8 ± 3                         | 8 ± 2       | 0.32 ± 0.01 | 0.32 ± 0.01 |

Final concentration of D-glucose is 5 mM. Control shows the data before glucose is added.



**Fig. 13.** Fluorescence emission spectra at 77 K in ambient [CO<sub>2</sub>] grown *S. 7002* (A) and *C. reinhardtii* (B). Cyanobacterial and algal cells were excited at 600 nm and 480 nm, respectively, and the spectra were normalized to the intensity of the fluorescence peak at 725 nm (PSI peak). The cells were sampled at CO<sub>2</sub>-saturated photosynthesis (black line), and CO<sub>2</sub>-limited photosynthesis (red line). The experiments were performed three times and the averaged data is shown.

#### 4.4. Discussion

The responses of photosynthetic electron transport system to CO<sub>2</sub>-limitation are rich in diversity in cyanobacteria and green algae. Previously, we reported that S. 7942 suppresses the photosynthetic electron transport system with a decrease in photosynthetic O<sub>2</sub> evolution, whereas S. 6803 expresses FLV2/4 and maintains Y(II) with O<sub>2</sub> as the alternative electron acceptor (Hayashi et al., 2014; Shimakawa et al., 2015). However, the marine cyanobacterium S. 7002 showed AEF during CO<sub>2</sub>-limited photosynthesis when the cells were grown under ambient [CO<sub>2</sub>] (Fig. 4), even though this strain does not have FLV2/4 encoding genes (Fujisawa et al., 2014; Fujisawa et al., 2017). We excluded O<sub>2</sub> in the reaction mixture by adding glucose oxidase, which suggested us that AEF in S. 7002 can utilize O<sub>2</sub> at extreme low concentration, compared with that in S. 6803 (Fig. 7 and 8). In other words, both S. 7002 and S. 6803 experience AEF during CO<sub>2</sub>-limited photosynthesis, but their underlying molecular mechanisms may differ. In sum, AEF during CO<sub>2</sub>-limited photosynthesis in cyanobacteria is not explained by the presence of FLV2/4 for all species. In addition, parallel to the AEF, NPQ was observed during CO<sub>2</sub>-limited photosynthesis in ambient [CO<sub>2</sub>] grown S. 7002 (Fig. 4). Measurements of Chl fluorescence spectra at a low temperature (77K) showed preferential excitation of PSI to PSII during CO<sub>2</sub>-limited photosynthesis in ambient [CO<sub>2</sub>] grown S. 7002 (Fig. 13), different from S. 6803 and S. 7942 (Miller et al., 1996; Hayashi et al., 2014). This suggests that the cyanobacterium S. 7002 utilizes a different strategy to alleviate or prevent oxidative damage under CO<sub>2</sub> limitation compared with S. 6803 and S. 7942. Interestingly, a model green alga *C. reinhardtii* showed similar physiological responses as ambient [CO<sub>2</sub>] grown S. 7002, including AEF and NPQ to the CO<sub>2</sub>-limited photosynthesis (Fig. 9–11). Additionally, in ambient [CO<sub>2</sub>] grown *C. reinhardtii*, some Y(II), estimated about 25% of that during CO<sub>2</sub>-saturated photosynthesis, does not require O<sub>2</sub> even where photosynthesis hardly

proceeds, implying that an O<sub>2</sub>-insensitive AEF partially functions as AEF replacing photosynthesis under CO<sub>2</sub>-limitation (Fig. 12). Below, we discuss the molecular mechanisms behind the AEF and the NPQ, as well as different low-[CO<sub>2</sub>] responses among cyanobacteria, and the evolutionary process of AEF in prokaryotic and eukaryotic algae.

Simultaneously measuring O<sub>2</sub> evolution and Chl fluorescence is a useful method to evaluate the activity of AEF not only in plant leaves, but also in prokaryotic and eukaryotic algae. Electron transport rates through PSII calculated from Y(II) have shown a proportional relationship with O<sub>2</sub> evolution rates and biomass yields in these algae (Campbell et al., 1998; Schuurmans et al., 2015). However, effects of cyanobacterial respiration (or chlororespiration) and the state transition on the Chl fluorescence signal are often raised as problems in the measurement in algae (Schuurmans et al., 2015). Nonetheless, the AEF method in this study was based on the data of Shimakawa et al. (2015) showing the positive linear relationship between O<sub>2</sub> evolution rate and Y(II) in *S. 7942* and the FLV2/4 mutant of *S. 6803* in the transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited photosynthesis. We further found that quantum yield of PSI, Y(I), is also kept in the transition to CO<sub>2</sub>-limited photosynthesis in ambient [CO<sub>2</sub>] grown *S. 7002* as well as Y(II), and decreases when O<sub>2</sub> in the medium is eliminated (see the “Part III”), which supports the data obtained by Chl fluorescence measurements in this study.

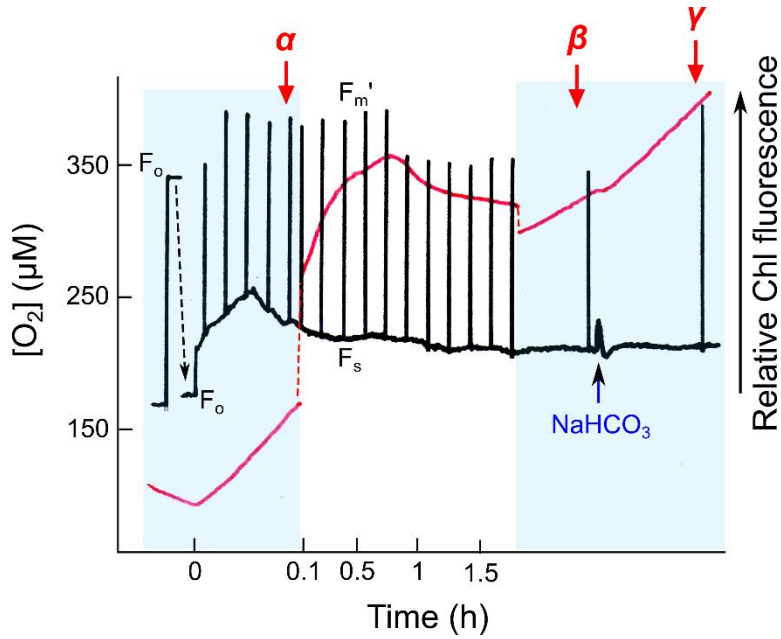
We developed several hypotheses to explain the molecular mechanism behind the O<sub>2</sub>-dependent AEF that replaces photosynthesis in *S. 7002* and *C. reinhardtii* during CO<sub>2</sub>-limited photosynthesis. Mehler reaction occurs in the acceptor side of PSI and drives the water-water cycle through the production and detoxification of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O (Mehler, 1951; Miyake and Asada, 2003; Roach et al., 2015), which is likely to have high affinity for O<sub>2</sub> ( $K_m$  is about 60–80 μM) (Miyake and Asada, 2003) than that of photorespiration ( $K_m$  is about 500 μM) (Jordan and Ogren, 1981). Additionally, FLV1/3 catalyze the reduction of O<sub>2</sub> directly to H<sub>2</sub>O in cyanobacteria (Mehler-like reaction) (Helman et al., 2003; Helman et al., 2005), and the  $K_m$

values of recombinant FLV proteins for O<sub>2</sub> is about 1–2 μM (Vicente et al., 2002; Vicente et al., 2008; Shimakawa et al., 2015). However, the contribution of FLV1/3 to AEF could not be observed during CO<sub>2</sub>-limited photosynthesis well in S. 6803 and S. 7942 (Hayashi et al., 2014; Shimakawa et al., 2015; Shaku et al., 2016), which is in agreement with the lower growth in the FLV2/4 mutants (Zhang et al., 2009; Zhang et al., 2012). Compared to FLV1/3, respiratory terminal oxidases can also mediate O<sub>2</sub>-dependent AEF under significantly low [O<sub>2</sub>], because of high affinities for O<sub>2</sub> (the *K<sub>m</sub>* values are about a few μM or less) (Berry et al., 2002; Beardall et al., 2003; Bernát and Rögner, 2011). In this study, we physiologically found the activity of an O<sub>2</sub>-dependent AEF replacing photosynthesis under CO<sub>2</sub>-limitation in S. 7002 and *C. reinhardtii*, both of which lack FLV2/4. The mutant study in these organisms should be addressed in future (see the “Part III”) (Chaux et al., 2017).

We found that parts of Y(II) uncoupled with photosynthesis during CO<sub>2</sub>-limited photosynthesis do not show the dependence on [O<sub>2</sub>] in S. 7002 and *C. reinhardtii* (Fig. 15 and 20), indicating that an O<sub>2</sub>-insensitive AEF functions in part. First, CEF around PSII, which is induced by a ΔpH across the thylakoid membranes, is assumed to be related to the O<sub>2</sub>-insensitive AEF. In the green alga *Chlorella pyrenoidosa*, an active cyclic electron flow around PSII was observed at light saturation and supraoptimal light intensities (Falkowski et al., 1986). When cyclic electron flow around PSII is active, Y(II) becomes uncoupled with photosynthetic O<sub>2</sub> evolution rates (Falkowski et al., 1986; Miyake and Yokota, 2001; Miyake et al., 2002). In addition, cyanobacteria have been known to utilize H<sup>+</sup> and nitrate, NO<sub>3</sub><sup>−</sup>, as alternative electron acceptors (Appel et al., 2000; Zhou et al., 2000; Flores et al., 2005). However, since the O<sub>2</sub> evolution rates decreased during CO<sub>2</sub>-limited photosynthesis, it would be remarkable if H<sup>+</sup> and NO<sub>3</sub><sup>−</sup> accepted electrons originating in H<sub>2</sub>O in PSII in ambient [CO<sub>2</sub>] grown S. 7002.

In ambient [CO<sub>2</sub>] grown S. 7002, NPQ was induced in the transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited photosynthesis (Fig. 12). In S. 6803 and S. 7942, *F<sub>m</sub>*' increased in this

transition, which did not lead to a change in excitation transfer efficiency between the antenna pigments, including state transition (Miller et al., 1996; Hayashi et al., 2014). However, in ambient [CO<sub>2</sub>] grown *S. 7002*,  $F_m'$  decreased with the transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited photosynthesis (Fig. 12), which is a first report showing increased NPQ under CO<sub>2</sub>-limitation in cyanobacterial species. The genomes of *S. 7002* and *S. 6803*, but not that of *S. 7942*, encode the genes for OCP. However, we have concluded that OCP is not related to the NPQ during CO<sub>2</sub>-limited photosynthesis in ambient [CO<sub>2</sub>] grown *S. 7002*, since we had applied red AL ( $620\text{ nm} < \lambda < 695\text{ nm}$ ) within the range of approximate growth light intensities. In fact, when we applied white AL ( $140\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ , Fig. 14), we obtained results similar to those obtained using red AL, which implies that OCP functions mainly under high-light conditions (Wilson et al., 2006). Additionally, photoinhibition of PSII also causes increased NPQ, but NPQ observed in *S. 7002* and *C. reinhardtii* were rapidly relaxed by adding NaHCO<sub>3</sub> (Fig. 4, 9, and 10). This suggests that the molecular mechanisms of the NPQ are not related to photoinhibition of PSII, which takes many minutes to hours for the relaxation (Murchie and Lawson, 2013). Furthermore, the 77 K Chl fluorescence spectra indicated a change in the distribution of excitation energy to two photosystems between CO<sub>2</sub>-saturated and CO<sub>2</sub>-limited photosynthesis in *C. reinhardtii* and ambient [CO<sub>2</sub>] grown *S. 7002* (Fig. 13). For eukaryotic green algae, several studies have reported a decrease in  $F_m'$  during CO<sub>2</sub>-limited photosynthesis, which was attributed to state transitions (Sültemeyer et al., 1989; Iwai et al., 2007; Ihnken et al., 2014). Our results suggest that the state transition may occur in the transition to CO<sub>2</sub>-limited photosynthesis in *C. reinhardtii* and ambient [CO<sub>2</sub>] grown *S. 7002*. In *C. reinhardtii*, a  $\Delta\text{pH}$ -inducible NPQ, which requires a stress-related light-harvesting complex protein known as LHCSR, has been known to be a dominant component of NPQ (Niyogi and Truong, 2013; Demmig-Adams et al., 2014). Next to a state transition, the  $\Delta\text{pH}$ -inducible NPQ might be involved in the NPQ during CO<sub>2</sub>-limited photosynthesis in *C. reinhardtii* (Kodru et al., 2015).



**Fig. 14.** The effect of white light on  $[O_2]$  (red) and relative Chl fluorescence (black) in ambient  $[CO_2]$  grown *S. 7002*. Initially media containing the cells ( $10 \mu\text{g Chl mL}^{-1}$ ) were kept dark; illumination with ML and white AL ( $140 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) started as indicated. The downward shift in  $F_0$  after the start of ML illumination is due to increasing the instrument amplification from 200 to 100 mV.  $NaHCO_3$  (final concentration 10 mM) was added as indicated. Indicated  $\alpha$ ,  $\beta$ , and  $\gamma$  denote times at which  $O_2$  evolution rates were determined. Blue shadings indicate that the oxygen electrode chamber was closed, the  $O_2$  signal amplitude was doubled, and measurement time scale was reduced to 1/10. Measurements were performed three times and representative data are shown.  $O_2$  evolution rates were  $-15 \pm 6$ ,  $50 \pm 4$ ,  $16 \pm 8$ ,  $55 \pm 8 \mu\text{mol } O_2 (\text{mg Chl})^{-1} \text{h}^{-1}$  respectively at dark,  $\alpha$ ,  $\beta$ , and  $\gamma$ .  $Y(II)$  were  $0.28 \pm 0.01$ ,  $0.26 \pm 0.01$ ,  $0.30 \pm 0.02$  respectively at  $\alpha$ ,  $\beta$ , and  $\gamma$  (mean  $\pm$  SD).

Cyanobacteria appear to acquire diverse physiological strategies to acclimate to low

[CO<sub>2</sub>] conditions. In this study, we first observed a *C. reinhardtii*-like response of photosynthetic electron transport system to CO<sub>2</sub>-limited photosynthesis in cyanobacteria, for which both an O<sub>2</sub>-dependent AEF, and NPQ originating from the change of PSII/PSI-excitation ratio simultaneously occur. Furthermore, this response was observed only when the cells were grown under ambient [CO<sub>2</sub>]. In contrast, the cells grown under high [CO<sub>2</sub>] displayed an *S. 7942*-like response to CO<sub>2</sub>-limited photosynthesis, with which *F<sub>s</sub>* increased and *Y(II)* decreased (Fig. 4) (Miller et al., 1996; Hayashi et al., 2014). In sum, a system induced by CO<sub>2</sub>-limitation might stimulate the O<sub>2</sub>-dependent AEF and NPQ in *S. 7002*. Furthermore, this system possibly exists in *S. 7002*, but not in *S. 7942*. Previously, we had assumed that the presence of FLV2/4 in cyanobacteria determines the responses of their photosynthetic electron transport systems to CO<sub>2</sub>-limitation (Shimakawa et al., 2015). However, *S. 7002* has provided a new insight into the physiological diversity of prokaryotic algae, even though this species is located adjacent to *S. 6803* in the phylogenetic trees, based on both small subunit rRNA sequence analyses (Turner et al., 1999) and average similarities of whole genome sequences (Satoh et al., 2013). On the other hand, RISE occurs under CO<sub>2</sub> limitation in the cyanobacterium *S. 7942* (Shaku et al., 2016). In addition, although the molecular mechanisms might differ, not only green algae but also vascular plants show NPQ under CO<sub>2</sub> limitation (Miyake et al., 2005), which suggests that these eukaryotic phototrophs have functionally evolved from the *S. 7002*-type cyanobacteria.

We note that photorespiration could occur, but would not have an electron flux capacity to function as an alternative electron sink in *C. reinhardtii* (Fig. 12). In previous physiological and biochemical studies, this alga has been found to exhibit photorespiration in the transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited photosynthesis before a CO<sub>2</sub>-concentrating mechanism is activated (Birmingham et al., 1982; Moroney et al., 1986; Marek and Spalding, 1991). However, the *Y(II)* during CO<sub>2</sub>-limited photosynthesis shows significantly high affinity for O<sub>2</sub> (Fig. 12), compared with the oxygenation reaction of Rubisco (Jordan and Ogren, 1981). This suggests

that photorespiration is not capable of accounting for all the excess light energy and cannot replace photosynthesis even if the photorespiratory C<sub>2</sub> cycle proceeds, similar to the cyanobacteria S. 6803, S. 7942, and S. 7002 (Fig. 7 and 8) (Hayashi et al., 2014; Shimakawa et al., 2015). On the other hand, in the C<sub>3</sub> plant tobacco, photorespiration does function as an alternative electron sink, replacing photosynthesis at the CO<sub>2</sub> compensation point (Driever and Baker, 2011; Sejima et al., 2016; Takagi et al., 2016). The photorespiratory C<sub>2</sub> cycle have already evolved at the origin of oxygenic phototrophs, which is likely to be as the scavenging system for 2-phosphoglycolate rather than an alternative energy sink (Eisenhut et al., 2008; Hagemann et al., 2013).

## Part II

# Diverse strategies of O<sub>2</sub> usage for preventing photo-oxidative damage under CO<sub>2</sub> limitation during algal photosynthesis

This part is based on the following publication:

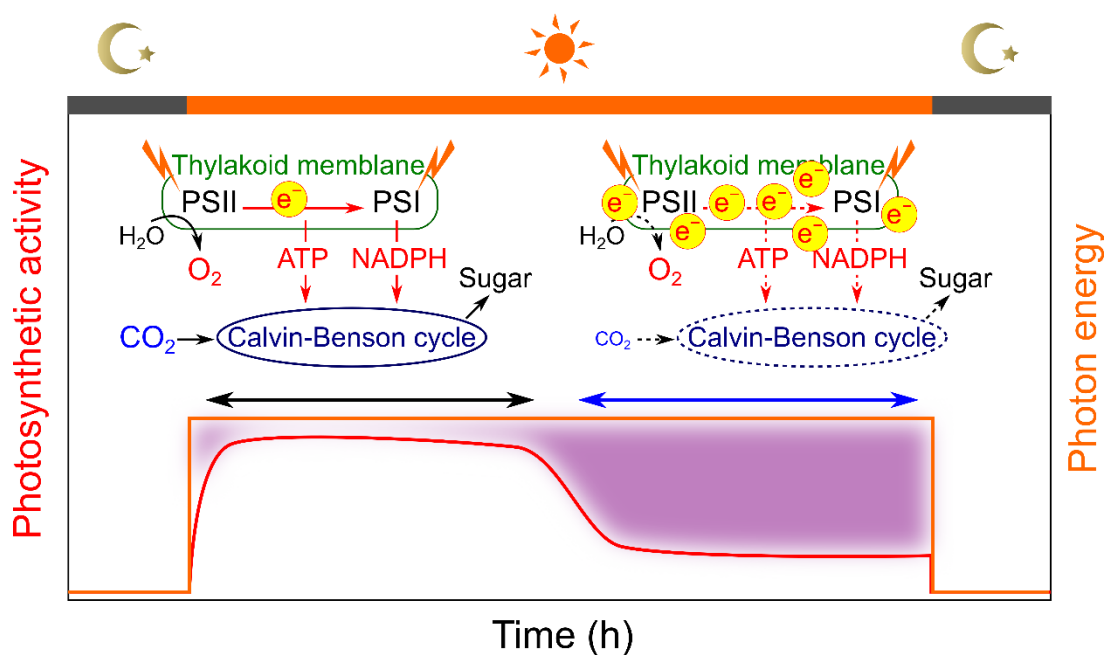
**Ginga Shimakawa, Yusuke Matsuda, Kensuke Nakajima, Masahiro Tamoi, Shigeru Shigeoka, Chikahiro Miyake** (2017) Diverse strategies of O<sub>2</sub> usage for preventing photo-oxidative damage under CO<sub>2</sub> limitation during algal photosynthesis. *Scientific Reports* 7 41022.

## 5.1. Abstract

Photosynthesis produces chemical energy from photon energy and assimilates CO<sub>2</sub> with the chemical energy. Thus, CO<sub>2</sub> limitation causes an accumulation of excess energy in the photosynthetic electron transport system, resulting in ROS which can cause oxidative damage to cells. O<sub>2</sub> can be used as an alternative energy sink when oxygenic phototrophs are exposed to high light. Here, we examined the responses to CO<sub>2</sub> limitation and O<sub>2</sub> dependency of two secondary algae, *Euglena gracilis* and *Phaeodactylum tricornutum*. In *E. gracilis*, approximately half of the relative ETR of CO<sub>2</sub>-saturated photosynthesis was maintained and was uncoupled from photosynthesis under CO<sub>2</sub> limitation. The ETR showed biphasic dependencies on O<sub>2</sub> at high and low O<sub>2</sub> concentrations. Conversely, in *P. tricornutum*, most relative ETR decreased in parallel with the photosynthetic O<sub>2</sub> evolution rate in response to CO<sub>2</sub> limitation. Instead, non-photochemical quenching was strongly activated under CO<sub>2</sub> limitation in *P. tricornutum*. The results indicate that these secondary algae adopt different strategies to acclimatize to CO<sub>2</sub> limitation, and that both strategies differ from those utilized by cyanobacteria and green algae. We summarize the diversity of strategies for prevention of photo-oxidative damage under CO<sub>2</sub> limitation in cyanobacterial and algal photosynthesis.

## 5.2. Introduction

The production and consumption of energy by photosynthetic electron transport and the Calvin-Benson cycle becomes unbalanced without sufficient  $\text{CO}_2$  ( $\text{CO}_2$ -limited photosynthesis; Fig. 15). Excess photon energy causes the production of ROS, which trigger oxidative damage to PSII and PSI, so-called photoinhibition (Kok, 1956; Krause et al., 1985; Inoue et al., 1989; Aro et al., 1993; Hideg et al., 1994; Terashima et al., 1994; Melis, 1999; Nishiyama et al., 2001; Nishiyama et al., 2004; Pospíšil et al., 2004; Murata et al., 2007; Tyystjärvi, 2008; Sonoike, 2011; Sejima et al., 2014; Tikkanen et al., 2014).



**Fig. 15.** A simple model of photosynthesis. Orange and red line shows photon energy and photosynthetic activity. Photosynthesis actively occurs when sufficient  $\text{CO}_2$  is available (double-headed black arrow). When available  $\text{CO}_2$  is not enough (double-headed blue arrow), excess photon energy accumulates in a photosynthetic electron transport system, leading to the production of ROS (indicated by a purple shading).

It is broadly accepted that O<sub>2</sub> is essential, not only as the respiratory electron acceptor, but also as an electron sink for various reactions of photosynthesis: O<sub>2</sub>-dependent AEF, including the water-water cycle (i.e., Mehler-ascorbate peroxidase pathway) (Miyake and Asada, 1992; Schreiber et al., 1995; Asada, 1999, 2000; Miyake and Asada, 2003), singlet O<sub>2</sub> production in PSII (Krieger-Liszkay, 2005), FLV reactions (Helman et al., 2003; Helman et al., 2005; Allahverdiyeva et al., 2011; Hayashi et al., 2014; Shimakawa et al., 2015), mitochondrial respiration (Weger et al., 1989; Dutilleul et al., 2003; Noguchi and Yoshida, 2008; Bailleul et al., 2015), and plastidial (or cyanobacterial) respiration (Pils et al., 1997; Pils and Schmetterer, 2001; Lea-Smith et al., 2013). Also, photorespiration can be explained as an O<sub>2</sub>-dependent AEF in the broad sense (Wiese et al., 1998; Driever and Baker, 2011; Sejima et al., 2016; Takagi et al., 2016; Hanawa et al., 2017). A large O<sub>2</sub>-dependent AEF that replaces photosynthesis can alleviate photoinhibition by dissipating excess energy to O<sub>2</sub> (Kozaki and Takeba, 1996; Allahverdiyeva et al., 2013; Bersanini et al., 2014; Hayashi et al., 2014; Shimakawa et al., 2015). Recently, we showed that an O<sub>2</sub>-dependent AEF is essential for the oxidation of P700 under CO<sub>2</sub> limitation to protect PSI against photo-oxidative damage in the cyanobacterium *S. 7002* (see the “Part III”). That is, oxygenic phototrophs accessed O<sub>2</sub> to prevent photo-oxidative damage derived from O<sub>2</sub>. However, both the magnitude and the molecular mechanisms of O<sub>2</sub>-dependent AEF vary across species in oxygenic phototrophs (Shimakawa et al., 2016) (see the “Part I”).

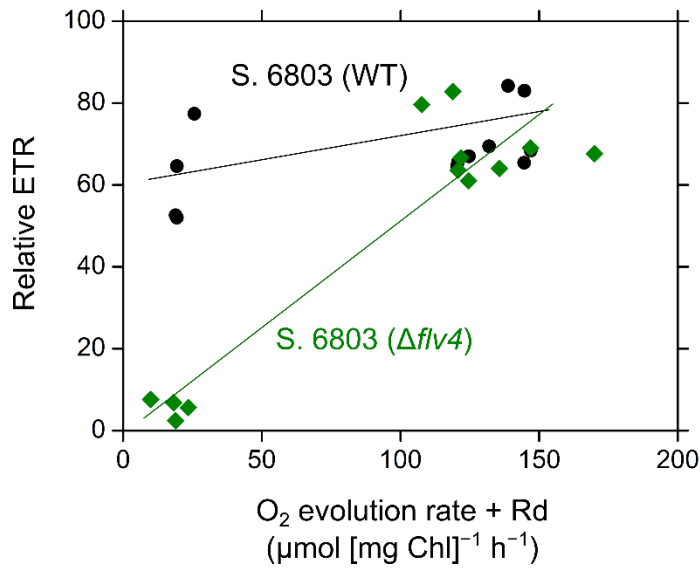
There are alternative mechanisms, which do not depend on O<sub>2</sub>, that dissipate excess photon energy in oxygenic phototrophs. First, during Chl fluorescence measurements, NPQ is observed as a decrease in maximum Chl fluorescence yields ( $F_m$  or  $F_m'$ ). Simply put, NPQ is a process of heat dissipation of photon energy around PSII. The molecular mechanisms of NPQ

in various oxygenic phototrophs are diverse and include the xanthophyll cycle, light-harvesting complex II aggregation, and state transition, some of which are activated by  $\Delta\text{pH}$  (Kanazawa and Kramer, 2002; Ruban et al., 2012; Derks et al., 2015; Naranjo et al., 2016). The degree of induced NPQ varies widely depending on growth and measurement conditions. Second, the electron transport in Cyt *b<sub>6</sub>/f* complex has suppressed sensitivity to  $\Delta\text{pH}$  (Tikhonov et al., 1981; Nishio and Whitmarsh, 1993; Kramer et al., 2003; Kramer et al., 2004; Takizawa et al., 2007) or reduced plastoquinone pool (Shaku et al., 2016), which is expected to oxidize PSI to alleviate the production of ROS by PSI owing to P700 quenching. Finally, CEF around PSI supports the formation of  $\Delta\text{pH}$  to induce NPQ and down-regulate the electron transport in Cyt *b<sub>6</sub>/f* (Nandha et al., 2007). We note that CEF is defined as an AEF but does not require  $\text{O}_2$ . In prokaryotic and eukaryotic algae, CEF around PSI is suggested to be driven in several pathways, including chloroplast NADPH dehydrogenase (NDH) 1 and 2, and an elusive ferredoxin-plastoquinone reductase (Shikanai, 2014; Yamori et al., 2016). Further, CEF around PSII has been found in the green alga *Chlorella pyrenoidosa* and isolated chloroplasts from spinach leaves (Falkowski et al., 1986; Miyake and Yokota, 2001; Miyake et al., 2002).

In this study, we measured responses to  $\text{CO}_2$  limitation of the cyanobacterium *S. 7942* and two secondary algae, the Euglenoid *Euglena gracilis* and the pennate marine diatom *Phaeodactylum tricornutum*. We aimed to elucidate the diversity of mechanisms to utilize  $\text{O}_2$  as an alternative electron acceptor in photosynthetic electron transport in cyanobacteria and algae. Cyanobacteria are known as the ancestors of chloroplasts, and have evolved into the chloroplasts of various photosynthetic eukaryotes via endosymbiosis. In contrast, the secondary algae are known to be the products of two endosymbiotic events and to have evolved from cyanobacteria along different lineages from that of land plants (Falkowski et al., 2004). Chloroplasts of *E. gracilis* are possibly derived from a green plastid-containing organism and are surrounded by a triple, rather than a double, membrane as found in vascular plants and green

algae, which possess Chl *a* and *b* as light-harvesting pigments. However, the relative content of Chl *b* to Chl *a* in *E. gracilis* is less than that in vascular plants. Conversely, *P. tricornutum* harbors chloroplasts that possibly originated from red algae, and are surrounded by a quadruple membrane. The light-harvesting complex of *P. tricornutum* has fucoxanthin-Chl *a/c* binding proteins containing the carotenoids diadinoxanthin and diatoxanthin, which are involved in NPQ (Cunningham and Schiff, 1986).

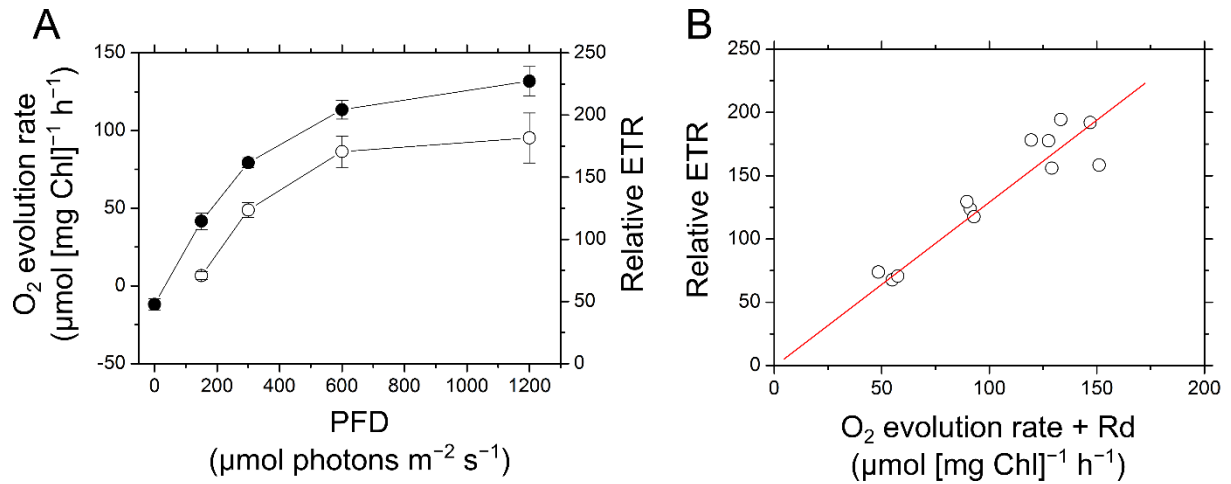
### 5.3. Results



**Fig. 16.** The relationship between gross photosynthetic O<sub>2</sub> evolution rate (net O<sub>2</sub> evolution rate + dark respiration rate [Rd]) and relative electron transport rate (ETR) in the cyanobacterium *S. 6803* WT and  $\Delta flv4$ . The data plotted were referred from our previous study (Shimakawa et al., 2015). Photosynthetic O<sub>2</sub> evolution rates and relative ETR were measured through the transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited conditions. Black circles, *S. 6803* WT; green diamonds,  $\Delta flv4$ . Cells in the reaction mixture (50 mM HEPES-KOH, pH 7.5, 10 μg Chl *a* mL<sup>-1</sup>) were illuminated with red actinic light (200 μmol photons m<sup>-2</sup> s<sup>-1</sup>).

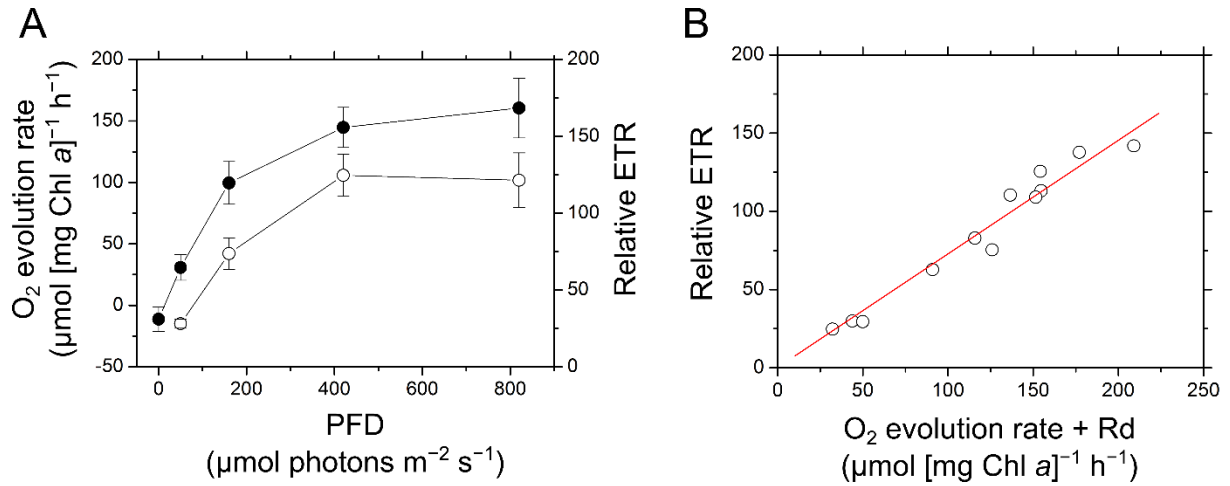
#### Responses of photosynthetic electron transport to CO<sub>2</sub> limitation in *S. 7942*, *E. gracilis*, and *P. tricornutum*

We measured O<sub>2</sub> and relative Chl fluorescence in *S. 7942*, *E. gracilis*, and *P. tricornutum* using O<sub>2</sub> electrode and a pulse-amplitude modification (PAM) fluorometer to evaluate the responses



**Fig. 17.** Light-response curve of  $\text{O}_2$  evolution rate and relative ETR during  $\text{CO}_2$ -saturated photosynthesis in *Euglena gracilis*. (A) Dependence of  $\text{O}_2$  evolution rate (closed circles) and relative ETR (open circles) on PFD. Negative values show Rd in the dark. Reaction media contained cyanobacterial cells ( $10 \mu\text{g Chl a mL}^{-1}$ ) and  $\text{NaHCO}_3$  (final concentration 10 mM). Measurements were independently taken three times, and the data are shown as means  $\pm$  SD. (B) The relationship between gross photosynthetic  $\text{O}_2$  evolution rate ( $\text{O}_2$  evolution rate + Rd) and relative ETR.

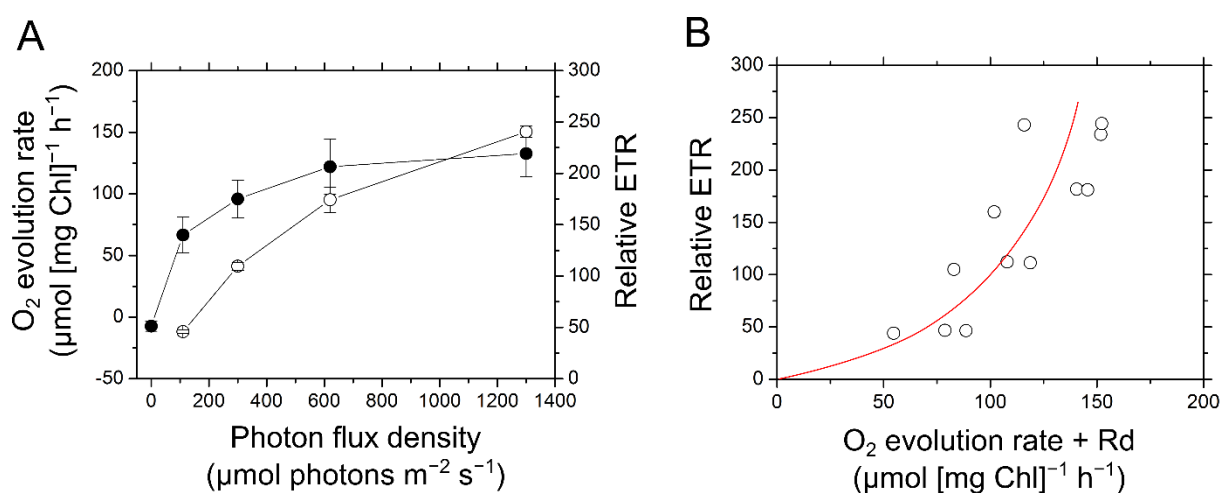
of photosynthetic electron transport. We estimated AEF activities in *S. 7942*, *E. gracilis*, and *P. tricornutum* using the relationship between photosynthetic  $\text{O}_2$  evolution rates and the relative ETR at PSII. Photosynthetic  $\text{O}_2$  evolution rate reflects the activity of photosynthesis (the Calvin-Benson cycle), whereas relative ETR is related to total electron transport activity, including AEF. Actually, we have found that the deletion of FLV2/4, which is the molecular mechanism of AEF under  $\text{CO}_2$  limitation, gives the proportional linear relationship between photosynthetic  $\text{O}_2$  evolution rates and relative ETR in the measurements using the wild type



**Fig. 18.** Light-response curve of O<sub>2</sub> evolution rate and relative ETR during CO<sub>2</sub>-saturated photosynthesis in *Phaeodactylum tricornutum*. (A) Dependence of O<sub>2</sub> evolution rate (closed circles) and relative ETR (open circles) on PFD. Negative values show Rd in the dark. Reaction media contained cyanobacterial cells (10 μg Chl a mL<sup>-1</sup>) and NaHCO<sub>3</sub> (final concentration 10 mM). Measurements were independently taken three times, and the data are shown as means ± SD. (B) The relationship between gross photosynthetic O<sub>2</sub> evolution rate (O<sub>2</sub> evolution rate + Rd) and relative ETR.

(WT) and the FLV4-knock-out mutant ( $\Delta flv4$ ) of *S. 6803* (Fig. 16) (Shimakawa et al., 2015). These rates showed proportional linearity in CO<sub>2</sub>-saturated conditions in the two secondary algae (Fig. 17 and 18), but not in *S. 7942* (Fig. 19), which suggests that electron transports at PSII were strongly coupled to photosynthesis in *E. gracilis* and *P. tricornutum* when sufficient CO<sub>2</sub> was available. In *S. 7942*, relative ETR was already partially uncoupled from photosynthetic O<sub>2</sub> evolution rate during CO<sub>2</sub>-saturated photosynthesis at supersaturated photon flux densities (Fig. 19), indicating that cyanobacterial AEF functions in such situations (Shaku

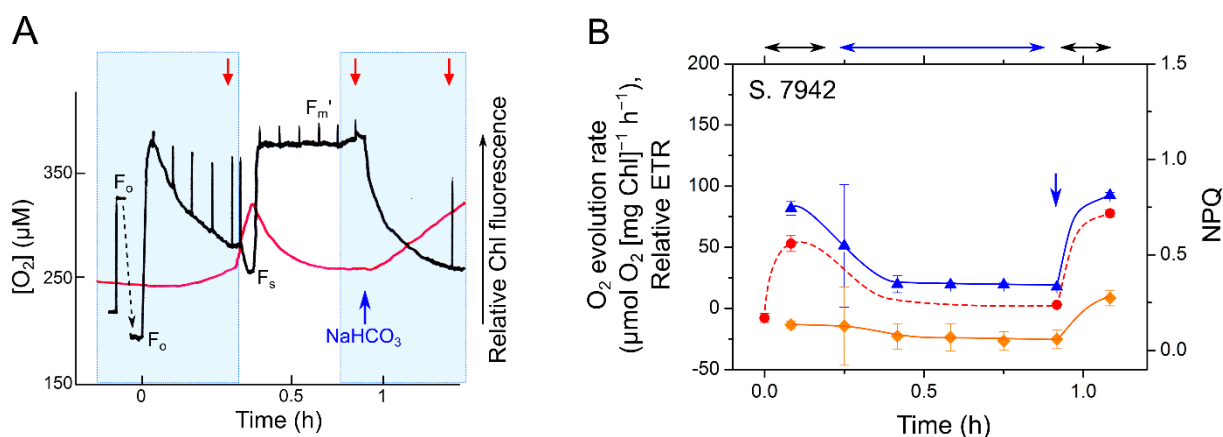
et al., 2016). We note that AEF can be reflected in the relative ETR only when the AEF has a high activity level comparable to photosynthesis.



**Fig. 19.** Light-response curve of O<sub>2</sub> evolution rate and relative ETR during CO<sub>2</sub>-saturated photosynthesis in *S. 7942*. (A) Dependence of O<sub>2</sub> evolution rate (closed circles) and relative ETR (open circles) on PFD. Negative values show Rd in the dark. Reaction media contained cyanobacterial cells (10  $\mu\text{g Chl a mL}^{-1}$ ) and NaHCO<sub>3</sub> (final concentration 10 mM). Measurements were independently taken three times, and the data are shown as means  $\pm$  SD. (B) The relationship between gross photosynthetic O<sub>2</sub> evolution rate (O<sub>2</sub> evolution rate + Rd) and relative ETR.

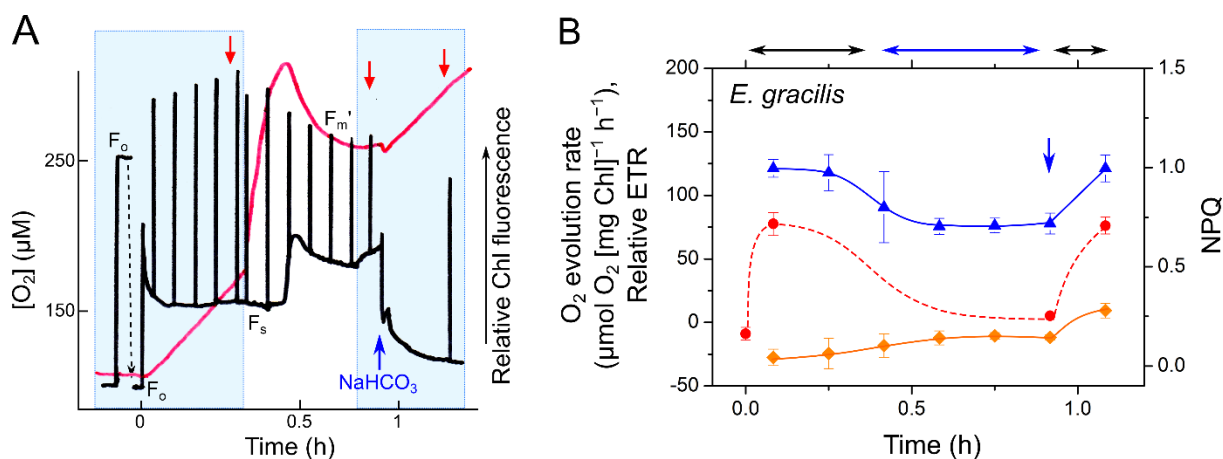
Responses of algal photosynthesis to CO<sub>2</sub> limitation were measured by following the method previously described (see the “Part I”) (Hayashi et al., 2014; Shimakawa et al., 2015). The responses of photosynthetic parameters to CO<sub>2</sub> limitation are shown in Fig. 20–22. The cyanobacterial and algal cells in fresh media were applied to an O<sub>2</sub> electrode chamber without adding inorganic carbon sources, and then illuminated with white AL. Illumination with AL

stimulated photosynthesis, which was accompanied by an increase in  $O_2$  in the reaction medium (Fig. 20–22). However,  $CO_2$  in the medium was gradually removed by algal photosynthesis, as the diffusion of  $CO_2$  from the atmosphere into the reaction medium was very slow, compared with the consumption by photosynthetic  $CO_2$  assimilation in the experimental system.  $O_2$  in the reaction medium began to decrease when  $CO_2$  was depleted (Fig. 20–22), indicating that photosynthesis was suppressed during the transition to  $CO_2$  limitation. The addition of  $CO_2$  (as  $NaHCO_3$ ) to the reaction medium restored photosynthetic activity (Fig. 20–22).



**Fig. 20.** Response of photosynthesis to  $CO_2$  limitation in *S. 7942*. (A) Raw trace showing the time course of  $O_2$  in the reaction media (red line) and relative Chl fluorescence of the cells (black line). Illumination with white AL ( $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) began at 0. Reaction media contained cyanobacterial cells ( $10 \mu\text{g Chl a mL}^{-1}$ ).  $NaHCO_3$  (final concentration 10 mM) was added at the point indicated by blue arrow. Blue shading indicates that the top of the  $O_2$  electrode chamber was closed and that the measurement time scales (x-axis) were reduced to 1/10.  $O_2$  evolution rates were determined at the times indicated by red arrows. Measurements were taken three times and representative data are shown. (B) Time courses of  $O_2$  evolution rate (red circles), relative ETR (blue triangles), and NPQ (orange diamonds). The double-headed black and blue arrows show  $CO_2$ -saturated and  $CO_2$ -limited photosynthesis, respectively. Data

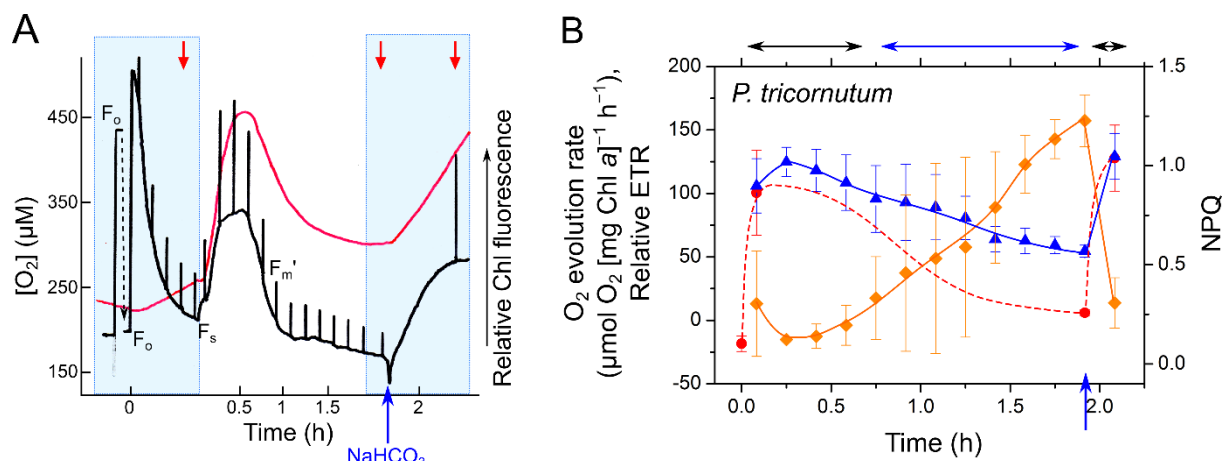
are shown as the mean  $\pm$  SD of three independent experiments.



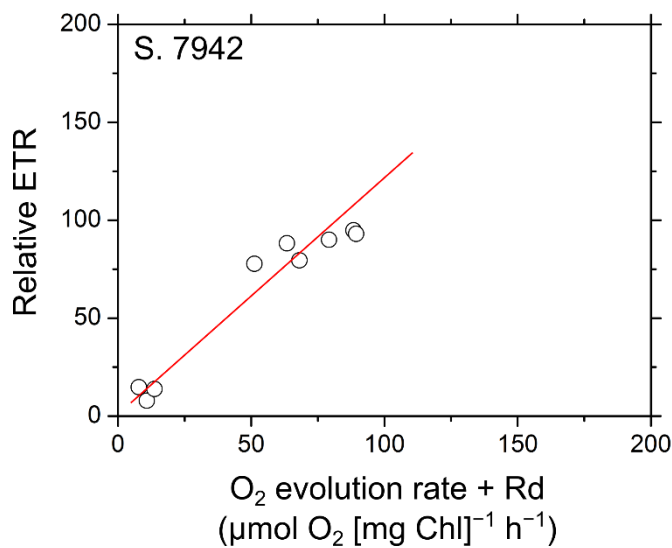
**Fig. 21.** Response of photosynthesis to CO<sub>2</sub> limitation in *E. gracilis*. (A) Raw trace showing the time course of O<sub>2</sub> in the reaction media (red line) and relative Chl fluorescence of the cells (black line). Illumination with white AL (300 μmol photons m<sup>-2</sup> s<sup>-1</sup>) began at 0. Reaction media contained cyanobacterial cells (10 μg Chl a mL<sup>-1</sup>). NaHCO<sub>3</sub> (final concentration 10 mM) was added at the point indicated by blue arrow. Blue shading indicates that the top of the O<sub>2</sub> electrode chamber was closed and that the measurement time scales (x-axis) were reduced to 1/10. O<sub>2</sub> evolution rates were determined at the times indicated by red arrows. Measurements were taken three times and representative data are shown. (B) Time courses of O<sub>2</sub> evolution rate (red circles), relative ETR (blue triangles), and NPQ (orange diamonds). The double-headed black and blue arrows show CO<sub>2</sub>-saturated and CO<sub>2</sub>-limited photosynthesis, respectively. Data are shown as the mean  $\pm$  SD of three independent experiments.

During the measurements, the top of the chamber remained open, which enabled O<sub>2</sub> and CO<sub>2</sub> to diffuse into or out of the reaction medium. This open system relieved excessive

increases in  $O_2$  in the reaction mixture, which enabled  $O_2$  to be measured for longer without passing over an undetectable point of the  $O_2$  electrode. We temporarily closed the chamber to exclude the effects of diffusion of  $O_2$  for determination of photosynthetic  $O_2$  evolution rates (Fig. 20–22) (Hayashi et al., 2014; Shimakawa et al., 2015; Shimakawa et al., 2016).

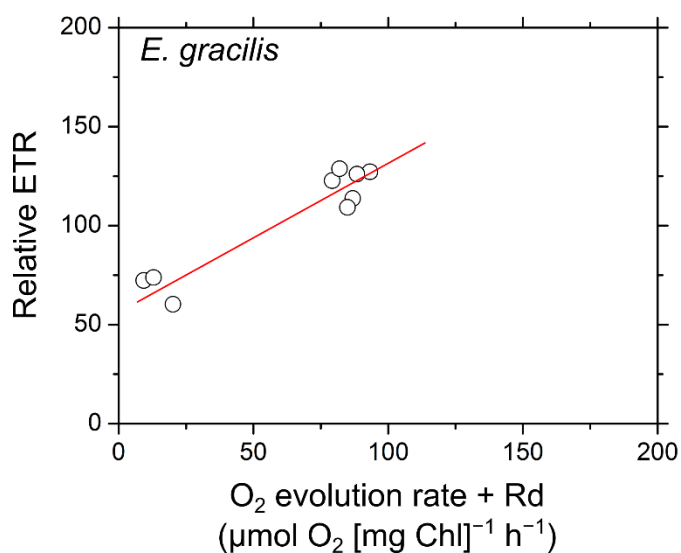


**Fig. 22.** Response of photosynthesis to  $CO_2$  limitation in *P. tricornutum*. (A) Raw trace showing the time course of  $O_2$  in the reaction media (red line) and relative Chl fluorescence of the cells (black line). Illumination with white AL ( $400 \mu mol photons m^{-2} s^{-1}$ ) began at 0. Reaction media contained cyanobacterial cells ( $10 \mu g Chl a mL^{-1}$ ).  $NaHCO_3$  (final concentration 10 mM) was added at the point indicated by blue arrow. Blue shading indicates that the top of the  $O_2$  electrode chamber was closed and that the measurement time scales (x-axis) were reduced to 1/10.  $O_2$  evolution rates were determined at the times indicated by red arrows. Measurements were taken three times and representative data are shown. (B) Time courses of  $O_2$  evolution rate (red circles), relative ETR (blue triangles), and NPQ (orange diamonds). The double-headed black and blue arrows show  $CO_2$ -saturated and  $CO_2$ -limited photosynthesis, respectively. Data are shown as the mean  $\pm$  SD of three independent experiments.

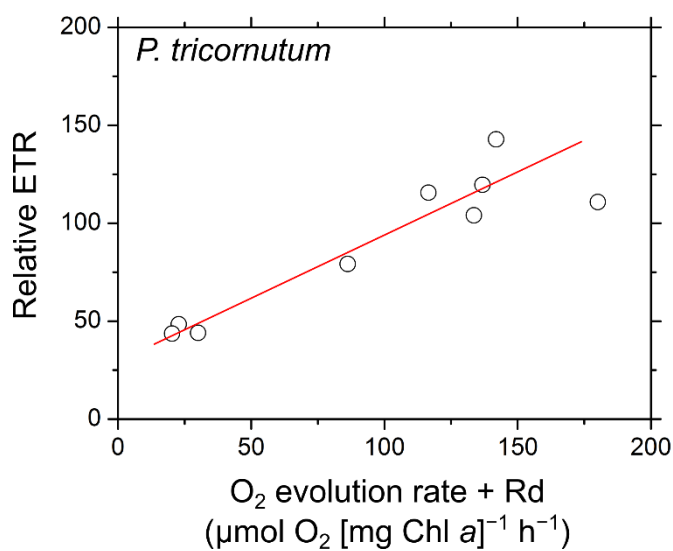


**Fig. 23.** The relationship between gross photosynthetic O<sub>2</sub> evolution rate (net O<sub>2</sub> evolution rate + Rd) and relative ETR in the three independent measurements in *S. 7942*.

In several earlier studies, it was observed that *S. 7942* induced little AEF or NPQ in response to CO<sub>2</sub> limitation (Miller et al., 1996; Hayashi et al., 2014; Shimakawa et al., 2015; Shaku et al., 2016). Thus, we used *S. 7942* as a control to compare the responses of *E. gracilis* and *P. tricornutum* in this study. In *S. 7942*, the photosynthetic O<sub>2</sub> evolution rate decreased in the transition to CO<sub>2</sub>-limited photosynthesis, which was paralleled by the decrease in the relative ETR without NPQ induction (Fig. 20). The proportional linear relationship between gross photosynthetic activity and relative ETR indicated that *S. 7942* hardly drives AEF in the transition to CO<sub>2</sub> limitation (Fig. 23). The increase in NPQ after adding NaHCO<sub>3</sub> has been observed in a previous study (Miller et al., 1996), although the molecular mechanism was unclear.



**Fig. 24.** The relationship between gross photosynthetic O<sub>2</sub> evolution rate (net O<sub>2</sub> evolution rate + Rd) and relative ETR in the three independent measurements in *E. gracilis*.



**Fig. 25.** The relationship between gross photosynthetic O<sub>2</sub> evolution rate (net O<sub>2</sub> evolution rate + Rd) and relative ETR in the three independent measurements in *P. tricornutum*.

In both secondary algae, particularly in *E. gracilis*, some relative ETR was uncoupled from the O<sub>2</sub> evolution rates during CO<sub>2</sub>-limited photosynthesis (Fig. 21, 22, 24, and 25), indicating that an AEF partially replaced photosynthesis during CO<sub>2</sub>-limited photosynthesis in these algae. Compared with S. 7942, *E. gracilis* maintained relative ETR uncoupled from the photosynthetic O<sub>2</sub> evolution rate during CO<sub>2</sub>-limited photosynthesis, which reached approximately half that during CO<sub>2</sub>-saturated photosynthesis (Fig. 21). Conversely, NPQ was slightly induced in the transition to CO<sub>2</sub> limitation in *E. gracilis*, which was not alleviated after at least 5 min after NaHCO<sub>3</sub> was added (Fig. 21). These results concurred with those of a previous study (Casper-Lindley and Björkman, 1998).

In the diatom *P. tricornutum*, a dramatic induction of NPQ was observed in the transition to CO<sub>2</sub> limitation with the suppression of photosynthesis, although the AEF activity was much less, compared with *E. gracilis* (Fig. 22). The relaxation of NPQ after adding NaHCO<sub>3</sub> was faster than that in *E. gracilis* (Fig. 22), which is in agreement with a number of studies of diatomaceous NPQ (Bailleul et al., 2010; Goss and Lepetit, 2015; Lepetit et al., 2016; Giovagnetti and Ruban, 2017). These data suggest that the NPQ in *E. gracilis* and *P. tricornutum* are derived from different molecular mechanisms.

### **Dependences of relative ETR on O<sub>2</sub> under CO<sub>2</sub> limitation in S. 7942, *E. gracilis*, and *P. tricornutum***

Diverse responses of photosynthetic electron transport to CO<sub>2</sub> limitation in S. 7942, *E. gracilis*, and *P. tricornutum* (Fig. 20–22) suggest different strategies of O<sub>2</sub> usage when photosynthesis is suppressed. To compare the O<sub>2</sub> usage of photosynthetic electron transport in these cyanobacterium and algae, we investigated the dependencies of relative ETR on O<sub>2</sub> during CO<sub>2</sub>-limited photosynthesis. We eliminated O<sub>2</sub> in the medium by adding glucose, catalase, and

glucose oxidase during CO<sub>2</sub>-limited photosynthesis using the method described by Shimakawa et al. (2016) (see the “Part I”). We confirmed in advance that the addition of exogenous glucose during illumination did not affect the O<sub>2</sub> evolution rates and relative ETR in these species (Table 1 and 3). The top of the O<sub>2</sub> electrode chamber was closed to exclude the effects of diffusion of O<sub>2</sub> and CO<sub>2</sub> into or out of the reaction medium in Fig. 26. It should be noted that there may have been some unintended consequences of using anaerobic conditions. However, removing O<sub>2</sub> did not affect relative ETR, at least during CO<sub>2</sub>-saturated photosynthesis, in *E. gracilis* or *P. tricornutum* (Fig. 27).

**Table 3.** Effects of D-glucose on photosynthesis in secondary algae

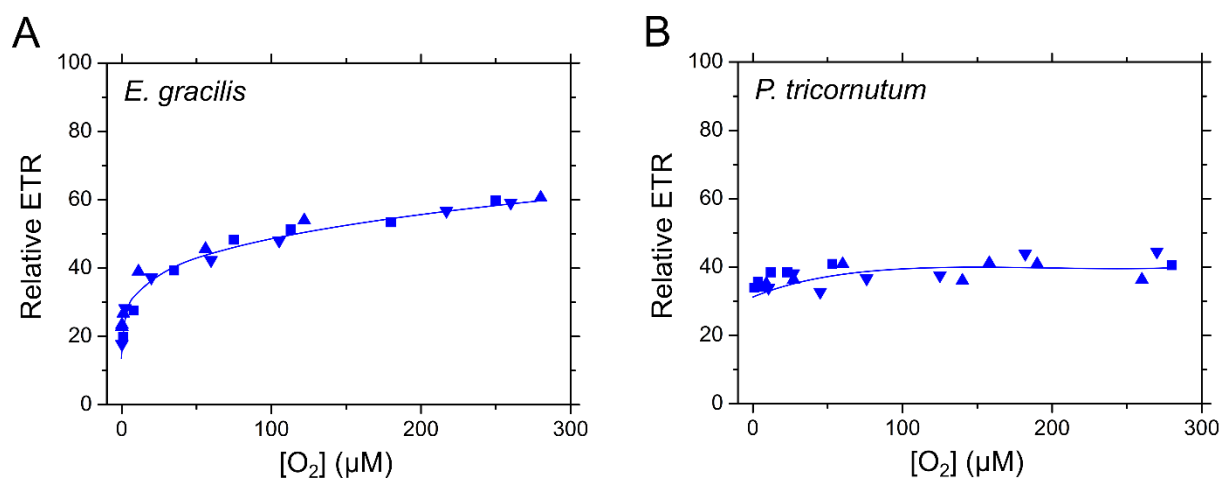
|                            | Control                                                                                     | + D-Glucose | Control      | + D-Glucose |
|----------------------------|---------------------------------------------------------------------------------------------|-------------|--------------|-------------|
|                            | O <sub>2</sub> evolution rate (μmol O <sub>2</sub> [mg Chl] <sup>-1</sup> h <sup>-1</sup> ) |             | Relative ETR |             |
| <i>E. gracilis</i>         |                                                                                             |             |              |             |
| CO <sub>2</sub> saturation | 85 ± 2                                                                                      | 86 ± 4      | 127 ± 3      | 127 ± 6     |
| CO <sub>2</sub> limitation | 8 ± 3                                                                                       | 7.1 ± 0.8   | 69 ± 5       | 69 ± 4      |
| <i>P. tricornutum</i>      |                                                                                             |             |              |             |
| CO <sub>2</sub> saturation | 127 ± 6                                                                                     | 130 ± 2     | 136 ± 7      | 138 ± 3     |
| CO <sub>2</sub> limitation | 3.0 ± 1.5                                                                                   | 3.1 ± 1.9   | 52 ± 3       | 50 ± 3      |

*Final concentration of D-glucose is 5 mM. Control shows the data before D-glucose is added.*

*Experiments under CO<sub>2</sub> saturation were conducted in the presence of 10 mM NaHCO<sub>3</sub>. Further, experiments were performed also under CO<sub>2</sub> limitation prepared by the analyses shown in Fig.*

*20–22. All of the other experimental procedures are the same as those in Fig. 20–22.*

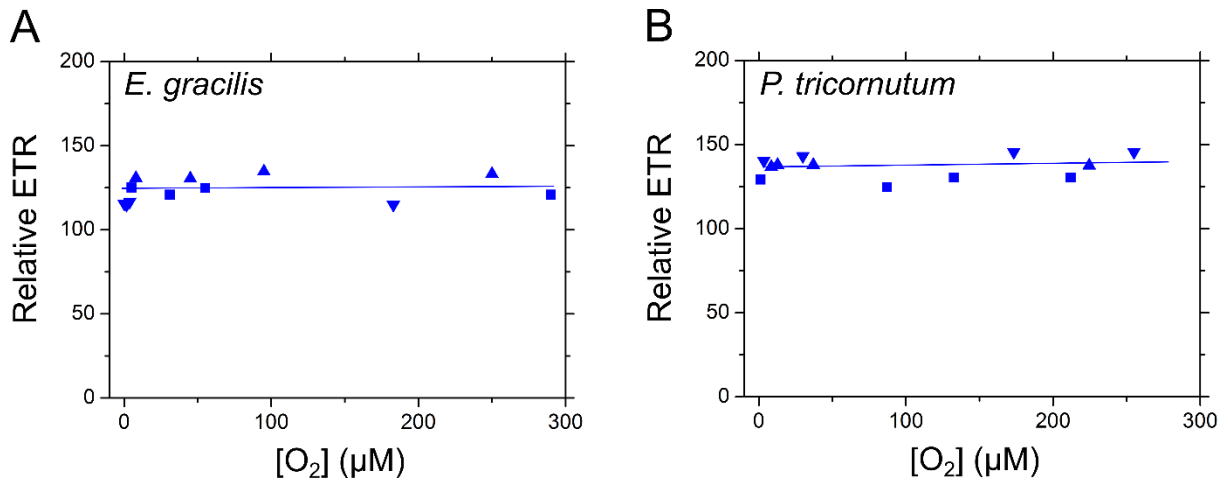
*Measurements were independently conducted three times, and the data are shown as means ± SD.*



**Fig. 26.** Dependence of relative ETR on  $O_2$  during  $CO_2$ -limited photosynthesis in *E. gracilis* (A) and *P. tricornutum* (B). To remove dissolved  $O_2$ , D-glucose (5 mM), catalase (250 units  $mL^{-1}$ ), and glucose oxidase (5 units  $mL^{-1}$ ) was added to the fresh media containing the cells ( $10 \mu g$  Chl  $a$   $mL^{-1}$ ). PFD of white AL were  $300 \mu mol$  photons  $m^{-2} s^{-1}$  for *E. gracilis*;  $400 \mu mol$  photons  $m^{-2} s^{-1}$  for *P. tricornutum*. Triangles, inverse triangles, and squares represent three independent measurements, respectively.

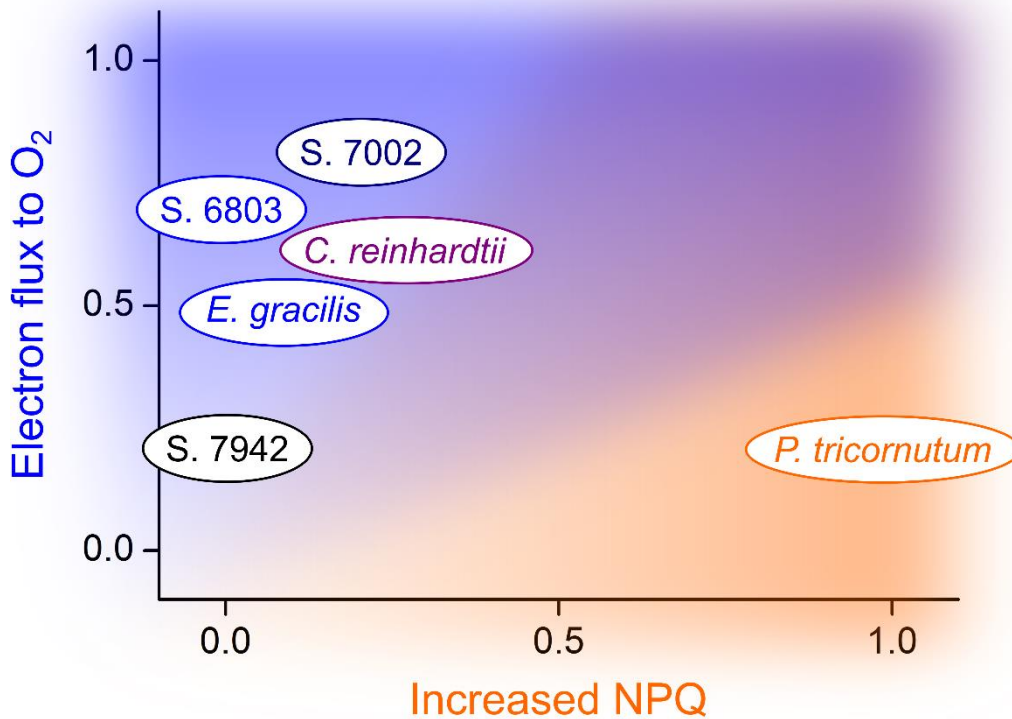
Compared with *S. 7942* and *P. tricornutum*, which required little  $O_2$  to drive AEF during  $CO_2$ -limited photosynthesis (Fig. 8 and 26B), *E. gracilis* showed a biphasic dependence on  $O_2$  (Fig. 26A). This indicated that more than two molecular mechanisms functioned as the  $O_2$ -dependent AEF in *E. gracilis*. Conversely, *P. tricornutum* was unlikely to rely on  $O_2$ -dependent AEF to alleviate photo-oxidative damage under  $CO_2$  limitation, compared with *E. gracilis*. There was residual relative ETR under anaerobic conditions in *E. gracilis* and *P. tricornutum*, which might be derived from an  $O_2$ -insensitive AEF, including CEF around PSI

and PSII (Wagner et al., 2016; Ananyev et al., 2017).



**Fig. 27.** Dependence of relative ETR on O<sub>2</sub> during CO<sub>2</sub>-saturated photosynthesis in *E. gracilis* (A) and *P. tricornutum* (B). To remove dissolved O<sub>2</sub>, D-glucose (5 mM), catalase (250 units mL<sup>-1</sup>), and glucose oxidase (5 units mL<sup>-1</sup>) was added to the fresh media containing the cells (10 μg Chl *a* mL<sup>-1</sup>). PFD of white AL were 300 μmol photons m<sup>-2</sup> s<sup>-1</sup> for *E. gracilis*; 400 μmol photons m<sup>-2</sup> s<sup>-1</sup> for *P. tricornutum*. Triangles, inverse triangles, and squares represent three independent measurements, respectively.

## 5.4. Discussion

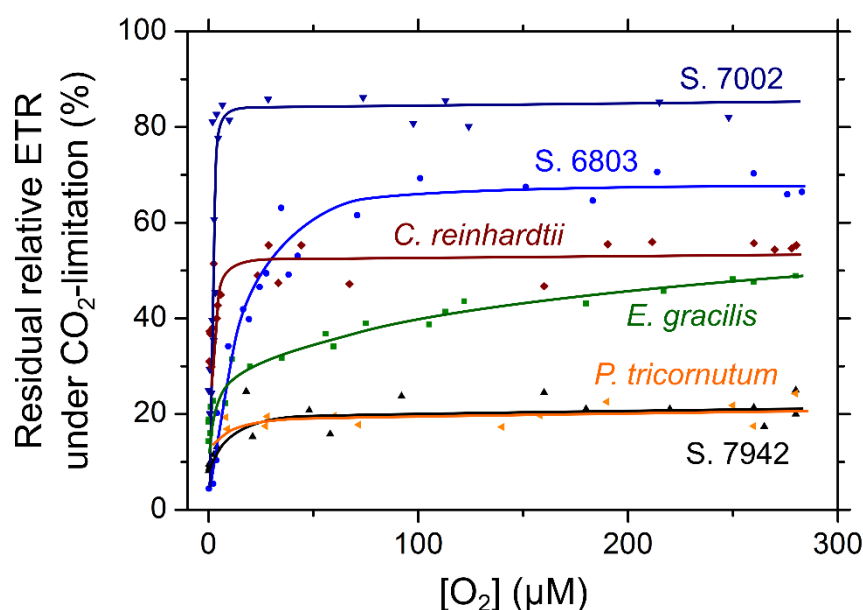


**Fig. 28.** A Summary diagram showing diverse responses of photosynthesis to CO<sub>2</sub> shortage. The plot is based on our previous and present results (Hayashi et al., 2014; Shimakawa et al., 2015; Shimakawa et al., 2016; Shimakawa et al., 2017).

Fig. 28 and 29 are summary diagrams of our previous and present results (Hayashi et al., 2014; Shimakawa et al., 2015; Shimakawa et al., 2016; Shimakawa et al., 2017) that presents the diverse responses of photosynthetic electron transport and O<sub>2</sub> usage to CO<sub>2</sub> limitation in cyanobacteria, green algae, and two classes of algae with secondary plastids. Oxygenic phototrophs possess a number of molecular mechanisms that protect their cells against photo-oxidative damage by ROS. In this study, we focused on the physiological significance of O<sub>2</sub> as

an alternative ‘safety valve’ in photosynthetic electron transport, and compared responses of photosynthesis to CO<sub>2</sub> limitation in genetically, phylogenetically, biologically, and ecologically different cyanobacteria and two classes of algae with secondary plastids. These organisms had different pigment compositions (Table 4), which made it difficult to quantitatively compare photosynthetic O<sub>2</sub> evolution rates and relative ETR. Therefore, we simply defined the ratio of relative ETR during CO<sub>2</sub>-limited photosynthesis to that during CO<sub>2</sub>-saturated photosynthesis as residual relative ETR under CO<sub>2</sub> limitation in each species, and summarized the dependencies on O<sub>2</sub> as shown in Fig. 29. The cyanobacterium S. 6803, harboring FLV2/4, showed the activation of an O<sub>2</sub>-dependent AEF during CO<sub>2</sub>-limited photosynthesis<sup>11</sup>. This was different from S. 7942, which does not possess FLV2/4. Conversely, the marine species S. 7002, which does not harbor FLV2/4, maintained a relatively high electron flux to O<sub>2</sub> during CO<sub>2</sub>-limited photosynthesis owing to the higher contribution of FLV1 and 3 homologs (i.e., FLV1/3) to AEF, compared with S. 7942 and S. 6803 (see the “Part III”). The green alga *C. reinhardtii* drives an O<sub>2</sub>-dependent AEF in the transition from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited photosynthesis, similar to S. 7002. The molecular mechanism of AEF in *C. reinhardtii* has recently been uncovered, and FLV1/3 mediate the dominant O<sub>2</sub>-dependent AEF also in the green alga (Chaux et al., 2017). The dependences of relative ETR on O<sub>2</sub> under CO<sub>2</sub> limitation in S. 6803, S. 7942, S. 7002, and *C. reinhardtii* have already been reported in Shimakawa et al. (2016) (see the “Part I”). In addition, in *E. gracilis*, the electron flux to O<sub>2</sub> partially replaced photosynthesis under CO<sub>2</sub> limitation, while the dependency on O<sub>2</sub> was different from that in S. 6803, S. 7002, and *C. reinhardtii*. The biphasic O<sub>2</sub> dependency of relative ETR in *E. gracilis* indicated that this alga might drive the other AEF, which has low affinity for O<sub>2</sub> (e.g., photorespiration) (Jordan and Ogren, 1981; Ogren, 1984) in addition to the AEF that has high O<sub>2</sub> affinity. Conversely, the diatom *P. tricornutum* hardly showed O<sub>2</sub> usage. Compared with cyanobacteria and algae, the C<sub>3</sub> plant tobacco mainly drives photorespiration as an O<sub>2</sub>-dependent AEF that replaces

photosynthesis at the CO<sub>2</sub> compensation point<sup>16</sup>, whereas this is not observed in the C<sub>4</sub> plant maize. Overall, there appear to be a number of diverse strategies of O<sub>2</sub> utilization that prevent photo-oxidative damage under CO<sub>2</sub> limitation, irrespective of the species of oxygenic phototroph, and O<sub>2</sub> is essential for some oxygenic phototrophs to protect cells against excess photon energy (Schreiber and Neubauer, 1990; Miyake, 2010).



**Fig. 29.** The diversity of O<sub>2</sub> usage strategies under CO<sub>2</sub> limitation in cyanobacterial and algal photosynthesis. Cyanobacterial and algal cells were grown under ambient CO<sub>2</sub>. Residual relative ETR under CO<sub>2</sub> limitation indicates the ratio of relative ETR during CO<sub>2</sub>-limited photosynthesis to that during CO<sub>2</sub>-saturated photosynthesis, which is shown with the dependency on O<sub>2</sub> (Hayashi et al., 2014; Shimakawa et al., 2016; Shimakawa et al., 2017).

Overall, dissipating photon energy to O<sub>2</sub> is not necessarily a universal strategy in oxygenic phototrophs during CO<sub>2</sub>-limited photosynthesis. In many oxygenic phototrophs, the

water-water cycle and respiratory terminal oxidases reduce O<sub>2</sub> at low concentrations, but in most cases, the rates estimated are less than 10% of CO<sub>2</sub>-saturated gross photosynthesis (Helman et al., 2005; Driever and Baker, 2011; Schuurmans et al., 2015; Shimakawa et al., 2015), whereas some species show high activity of water-water cycle *in vivo* (Waring et al., 2010). In this study, we measured the activities of the water-water cycle under CO<sub>2</sub> limitation in *S. 7942*, *E. gracilis*, and *P. tricornutum* by adding exogenous H<sub>2</sub>O<sub>2</sub>. The maximum activities reached approximately 70%, 25%, and 10% of the gross photosynthetic O<sub>2</sub>-evolution rates in *S. 7942*, *E. gracilis*, and *P. tricornutum*, respectively (Fig. 30). These estimates can be applied to the dependence of relative ETR on O<sub>2</sub> under CO<sub>2</sub> limitation in *E. gracilis*, but not in *S. 7942* or *P. tricornutum*. That is, both *S. 7942* and *P. tricornutum* probably suppressed the water-water cycle under CO<sub>2</sub> limitation, and relied upon alternative strategies to dissipate excess photon energy.

**Table 4.** Nitrogen-based Chl contents in cyanobacteria and algae used in this study

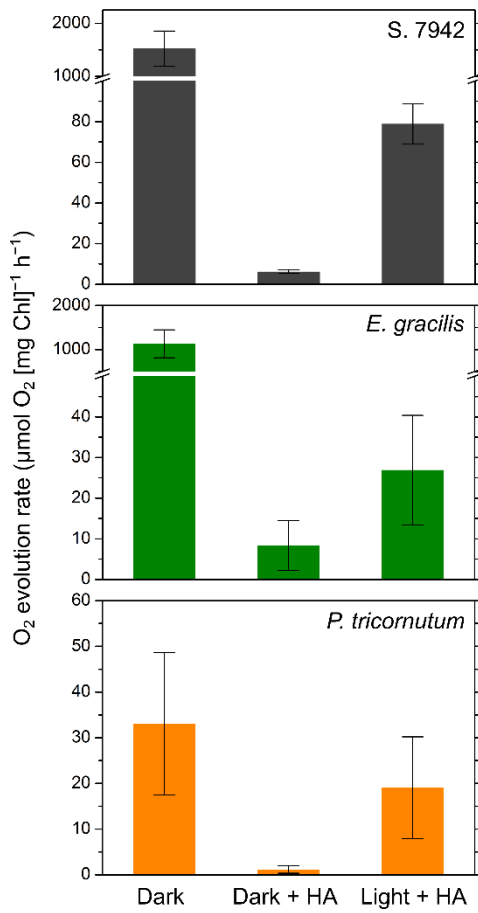
|                       | Chl content (mg Chl mg N <sup>-1</sup> ) |              |               |                                                   |
|-----------------------|------------------------------------------|--------------|---------------|---------------------------------------------------|
|                       | Total Chl                                | Chl <i>a</i> | Chl <i>b</i>  | Chl <i>c</i> <sub>1</sub> + <i>c</i> <sub>2</sub> |
| <i>S. 7942</i>        | 0.19 ± 0.04                              | 0.19 ± 0.04  | -             | -                                                 |
| <i>E. gracilis</i>    | 0.36 ± 0.03                              | 0.32 ± 0.04  | 0.040 ± 0.014 | -                                                 |
| <i>P. tricornutum</i> | 0.34 ± 0.04                              | 0.31 ± 0.04  | -             | 0.035 ± 0.003                                     |

*Data are shown as means ± SD of three independent experiments.*

There are mechanisms other than O<sub>2</sub>-dependent AEF that function in the protection of cells against photo-oxidative damage, which would explain why there are diverse strategies of

O<sub>2</sub> usage in oxygenic phototrophs (Shimakawa et al., 2015; Sejima et al., 2016; Shaku et al., 2016; Shimakawa et al., 2016; Shimakawa et al., 2016; Shimakawa et al., 2017). Increased NPQ is broadly used in many oxygenic phototrophs to dissipate excess photon energy, but the molecular mechanisms are unlikely to have the same origin. In the cyanobacterium S. 6803, it was observed that OCP was expressed and functioned in NPQ in response to high light levels (Hihara et al., 2001; Wilson et al., 2006). However, in the transition to CO<sub>2</sub> limitation, no induction of NPQ was observed in S. 7942 or S. 6803. The strategy to enhance NPQ under CO<sub>2</sub> limitation might not have been widespread in oxygenic phototrophs during their early evolution. The diatom *P. tricornutum* showed a large increase in NPQ under CO<sub>2</sub> limitation (Fig. 22), which would be strictly related to  $\Delta$ pH, some carotenoids, and the gene product of *lhcx*s (Bailleul et al., 2010; Goss and Lepetit, 2015; Lepetit et al., 2016). Conversely, the suppression of electron transport in Cyt *b<sub>6</sub>/f* is stimulated by  $\Delta$ pH (Tikhonov et al., 1981; Nishio and Whitmarsh, 1993; Kramer et al., 2003; Kramer et al., 2004; Takizawa et al., 2007; Takizawa et al., 2008; Tikhonov, 2012; Kohzuma et al., 2013; Tikhonov, 2013; Schöttler and Tóth, 2014; Tikhonov, 2014; Schöttler et al., 2015; Wang et al., 2015) and the reduction of the PQ pool (Shaku et al., 2016), both of which can cause the oxidation of P700 to dissipate excess photon energy. Additionally, O<sub>2</sub>-insensitive AEF, including CEF around PSI (Arnon et al., 1954; Endo et al., 1998; Yamamoto et al., 2006; Shikanai, 2014, 2016) and PSII (Falkowski et al., 1986; Miyake and Yokota, 2001; Miyake et al., 2002) may function to alleviate photo-oxidative damage. Furthermore, phototaxis possibly functions as a main strategy to avoid excess photon energy under CO<sub>2</sub> limitation in motile algae, such as *E. gracilis* (Richter et al., 2002; Richter et al., 2007). These O<sub>2</sub>-insensitive strategies to alleviate photo-oxidative damage would enable various oxygenic phototrophs to be independent of O<sub>2</sub>-dependent AEF. Nevertheless, the questions of the benefit (or cost) of O<sub>2</sub> usage to dissipate excess photon energy remains. There are still many questions over the diverse strategies that oxygenic phototrophs use to counter the

detrimental effects of sunlight.



**Fig. 30.**  $H_2O_2$ -induced  $O_2$  evolution in *S. 7942*, *E. gracilis*, and *P. tricornutum*.  $H_2O_2$  (0.5 mM) was added to the reaction media containing cyanobacterial or algal cells ( $10 \mu g \text{ Chl a mL}^{-1}$ ) in the dark and during  $CO_2$ -limited photosynthesis. PFD of white AL were  $300 \mu mol \text{ photons m}^{-2} \text{ s}^{-1}$  for *S. 7942* and *E. gracilis* and  $400 \mu mol \text{ photons m}^{-2} \text{ s}^{-1}$  for *P. tricornutum*. To exclude  $O_2$  evolution by catalase reaction, we added hydroxylamine (HA) at 0.1 mM for *S. 7942* and *P. tricornutum*, and 0.5 mM for *E. gracilis*. For the measurements during  $CO_2$ -limited photosynthesis, we added  $H_2O_2$  after photosynthetic  $O_2$  evolution rate reduced to almost 0, with the top of the  $O_2$  electrode chamber closed. Measurements were independently taken three times, and the data are shown as means  $\pm$  SD.

## Part III

# Oxidation of P700 in photosystem I is essential for the growth of cyanobacteria

This part is based on the following publication:

**Ginga Shimakawa, Keiichiro Shaku, Chikahiro Miyake** (2016) Oxidation of P700 in photosystem I is essential for the growth of cyanobacteria. *Plant Physiology* 172(3) 1443–1450.

## 6.1. Abstract

The photoinhibition of PSI is lethal to oxygenic phototrophs. Nevertheless, it is unclear how photo-damage occurs or how oxygenic phototrophs prevent it. Here, we provide evidence that keeping P700 oxidized protects PSI. Previous studies have suggested that PSI photoinhibition does not occur in the two model cyanobacteria, S. 6803 and S. 7942, when photosynthetic CO<sub>2</sub> fixation was suppressed under low CO<sub>2</sub> partial pressure even in mutants deficient in FLV, which mediates AEF. The lack of FLV in S. 7002, however, is directly linked to reduced growth and PSI photo-damage under CO<sub>2</sub> limiting conditions. Unlike S. 6803 and S. 7942, S. 7002 reduced P700 during CO<sub>2</sub>-limited illumination in the absence of FLV, resulting in the decreases in both PSI and photosynthetic activities. Even at normal air [CO<sub>2</sub>], the growth of S. 7002 mutant was retarded relative to that of the WT. Therefore, P700 oxidation is essential for protecting PSI against photoinhibition. In the Part III, we present various strategies to alleviate PSI photoinhibition in cyanobacteria.

## 6.2. Introduction

Low CO<sub>2</sub> 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 CO<sub>2</sub> limitation increase NADPH and ATP levels beyond the Calvin-Benson cycle requirements. Electrons and H<sup>+</sup> accumulate in the photosynthetic electron transport system. Excess electrons in the photosynthetic electron transport system triggers oxidative damage to PSI by forming ROS, including O<sub>2</sub><sup>-</sup> and <sup>1</sup>O<sub>2</sub> within PSI and inactivating the photochemical activity of PSI (Sato, 1970; Terashima et al., 1994; Sonoike et al., 1995; Sonoike, 2011; Cazzaniga et al., 2012; Sejima et al., 2014; Zivcak et al., 2015; Zivcak et al., 2015; Cazzaniga et al., 2016; Takagi et al., 2016). PSI repair has been reported to be a very slow process (Kudoh and Sonoike, 2002), and a recent study showed that it took more than 12 days for damaged PSI in wheat leaves to recover completely (Zivcak et al., 2015). PSI photoinhibition is, therefore, very detrimental to oxygenic phototroph growth. Nevertheless, PSI photoinhibition is alleviated by keeping P700 oxidized (Sejima et al., 2014).

In the photosynthetic electron transport system of oxygenic phototrophs, P700 oxidation is a physiological response to environmental variations. In C<sub>3</sub> plants, low CO<sub>2</sub> and/or high light intensity induce P700 oxidation *in vivo* (Badger and Schreiber, 1993; Klughammer and Schreiber, 1994; Laisk and Oja, 1994; Oja et al., 2003; Miyake et al., 2004; Miyake et al., 2005; Schreiber and Klughammer, 2008). Several molecular mechanisms are proposed for P700 oxidation wherein the PSI acceptor does not limit the photosynthetic electron transport reaction. First, H<sup>+</sup> accumulation on the lumenal side of thylakoid membranes lowers PQH<sub>2</sub> oxidation rates in Cyt *b<sub>6</sub>/f* (Kramer et al., 1999). Second, PTOX and cyanobacterial RTOs on the thylakoid membranes suppress PSI electron influx by accepting upstream PSI electrons in the photosynthetic electron transport system. O<sub>2</sub> is the final electron acceptor (Trouillard et al.,

2012; Mullineaux, 2014, 2014). Finally, PQH<sub>2</sub> accumulation inhibits the Q-cycle turnover in Cyt *b<sub>6</sub>/f*, which suppresses electron flow from Cyt *b<sub>6</sub>/f* to P700. This reaction is called RISE (Shaku et al., 2016). Overall, these molecular mechanisms contribute to P700 oxidation, thereby preventing PSI photoinhibition and enabling oxygenic phototrophs to thrive. The *pgr5* mutant of *Arabidopsis thaliana* cannot keep P700 oxidized and shows PSI photoinhibition under high- and fluctuating light conditions (Munekage et al., 2002; Munekage et al., 2004; Suorsa et al., 2012; Suorsa et al., 2013; Kono et al., 2014; Kono and Terashima, 2016), which shows an 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 FLV could contribute to P700 oxidation. Four FLV isozymes (FLV1–4) have been identified in the model cyanobacterium *S. 6803* (Helman et al., 2003). FLV1 and FLV3 (i.e., FLV1/3) function as a heterodimer and catalyze the reduction of O<sub>2</sub> to H<sub>2</sub>O on the acceptor side of PSI using NAD(P)H as the electron donors (Vicente et al., 2002; Helman et al., 2003; Helman et al., 2005; Allahverdiyeva et al., 2011; Allahverdiyeva et al., 2013; Allahverdiyeva et al., 2015). Unlike FLV1/3, FLV2/4 is induced only under low CO<sub>2</sub> (Zhang et al., 2009; Eisenhut et al., 2012), and mediates an O<sub>2</sub>-dependent AEF (Hayashi et al., 2014; Shimakawa et al., 2015). In *S. 6803*, FLV-dependent electron fluxes are coupled to photosynthesis and should alleviate electron over-accumulation in PSI (Helman et al., 2003; Allahverdiyeva et al., 2013; Shimakawa et al., 2015). Therefore, FLV is expected to contribute to P700 oxidation. The lack of FLV1/3 in *S. 6803* causes PSI photoinhibition under artificial fluctuating light (Allahverdiyeva et al., 2013). However, under CO<sub>2</sub> limitation (which suppresses photosynthetic CO<sub>2</sub> fixation), deletions of FLV1/3 and FLV2/4 do not cause PSI photoinhibition in *S. 6803* (Zhang et al., 2009) or *S. 7942* (Shaku et al., 2016), possibly because P700 stays oxidized under CO<sub>2</sub> limitation regardless of the existence of FLV. These data imply that FLV is not essential to keep P700 oxidized under CO<sub>2</sub> limitation, at least in *S. 6803* and *S. 7942*.

In the present study, we found that the lack of FLV1/3 leads to growth inhibition under ambient [CO<sub>2</sub>] in the cyanobacterium S. 7002, unlike S. 6803 and S. 7942. The S. 7002 genome, like that of S. 7942, includes genes coding for FLV1/3 isozymes but not for FLV2/4 (Fujisawa et al., 2014; Fujisawa et al., 2017). The genetic profiles of *flv* and other genes related to cyanobacterial AEF, including those of S. 7002, S. 6803, and S. 7942, are summarized in Table 5. Shifting from CO<sub>2</sub>-saturated to CO<sub>2</sub>-limited conditions decreased total oxidizable P700 to approximately 10% in the *flv*-knockout mutant of S. 7002, but not in those of S. 6803 or S. 7942. We demonstrated 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 photo-oxidative damage under CO<sub>2</sub> limitation.

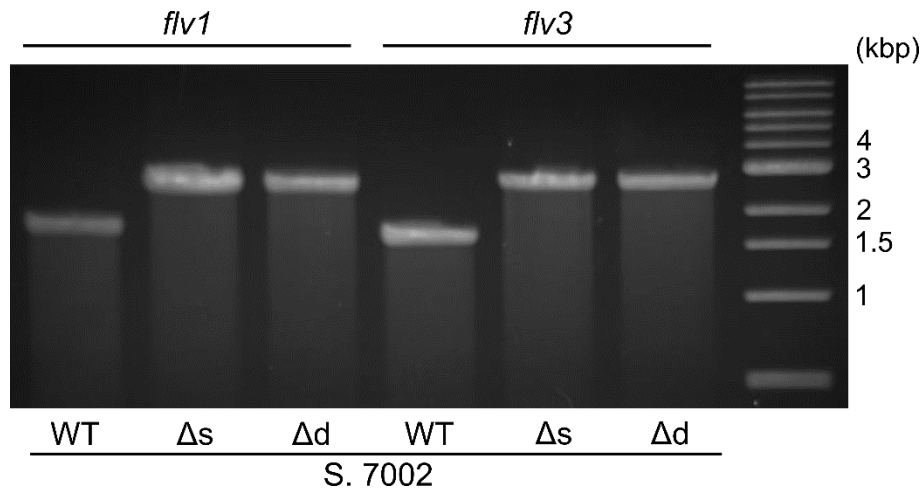
**Table 5.** Genetic background of AEF in three cyanobacteria species used in the “Part III”

|         | FLV           |                | RTOs             |                | CEF            |                |                |
|---------|---------------|----------------|------------------|----------------|----------------|----------------|----------------|
|         | <i>flv1/3</i> | <i>flv2/4</i>  | <i>cox</i>       | <i>cyd</i>     | <i>arto</i>    | <i>ndhD1/2</i> | <i>pgr5</i>    |
| S. 7002 | A1743         | –              | A1162            | –              | A0725          | A2000          | A1477          |
|         | /A1321        |                | –A1164           |                | –A0727         | /A1973         |                |
| S. 6803 | <i>sl1521</i> | <i>sl10219</i> | <i>slr1136</i>   | <i>slr1379</i> | <i>slr2082</i> | <i>slr0331</i> | <i>ssr2016</i> |
|         | /sl10550      | /sl10217       | – <i>slr1138</i> | /slr1380       | /slr2083       | /slr1291       |                |
|         |               |                |                  |                | /sl10813       |                |                |
| S. 7942 | 1810          | –              | 2602             | 1766           | –              | 1976           | –              |
|         | /1809         |                | –2604            | /1767          |                | /1439          |                |

Gene homology analyses were performed in cyanobase (<http://genome.microbedb.jp/CyanoBase>).

*cox*, aa<sub>3</sub>-type cytochrome c oxidase; *cyd*, cytochrome bd-type quinol oxidase; *arto*, cytochrome b<sub>o</sub>-type quinol oxidase; *ndhD*, D subunit of NAD(P)H dehydrogenase; *pgr5*, proton gradient regulation 5.

### 6.3. Results

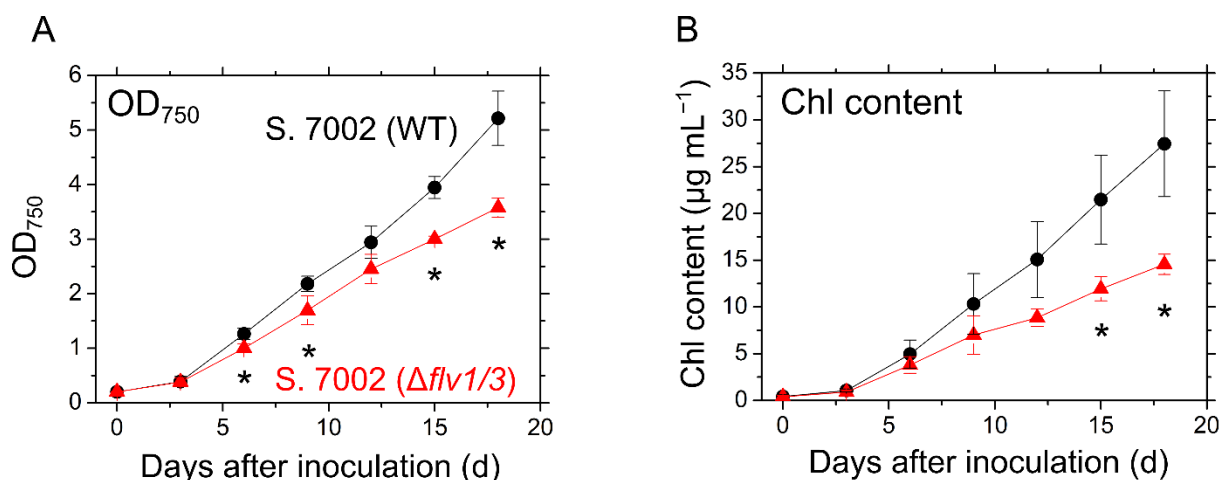


**Fig. 31.** Insertional inactivation of *flv* genes in *S. 7002*. DNA fragments amplified by PCR showing complete segregation of the inactivated genes, *flv1* (SYNPCC7002\_A1743) and *flv3* (SYNPCC7002\_A1321). WT, *S. 7002* WT;  $\Delta s$ , single mutants of *S. 7002* ( $\Delta flv1$  and  $\Delta flv3$ );  $\Delta d$ , the double mutant of *S. 7002* ( $\Delta flv1/3$ ).

#### Effects of FLV on the growth of *S. 7002* under ambient [CO<sub>2</sub>]

We constructed the *S. 7002* mutant,  $\Delta flv1/3$ , which lacks the *flv1* and *flv3* orthologs present in *S. 6803* (SYNPCC7002\_A1743 and SYNPCC7002\_A1321; Fig. 31). We found that the growth of  $\Delta flv1/3$  of *S. 7002* is slower than that of WT under ambient [CO<sub>2</sub>] (Fig. 32). This response was not observed in either *S. 6803* (Zhang et al., 2009; Zhang et al., 2012) or *S. 7942* (Shaku et al., 2016). Approximately 2 weeks after inoculation, the optical density at 750 nm (i.e., OD<sub>750</sub>) for  $\Delta flv1/3$  was 70% lower than that for the WT (Fig. 32A). The Chl content in the  $\Delta flv1/3$  media was half that of WT (Fig. 32B). These results indicate that *S. 7002* requires

FLV1/3 for optimal growth under ambient  $[\text{CO}_2]$ .

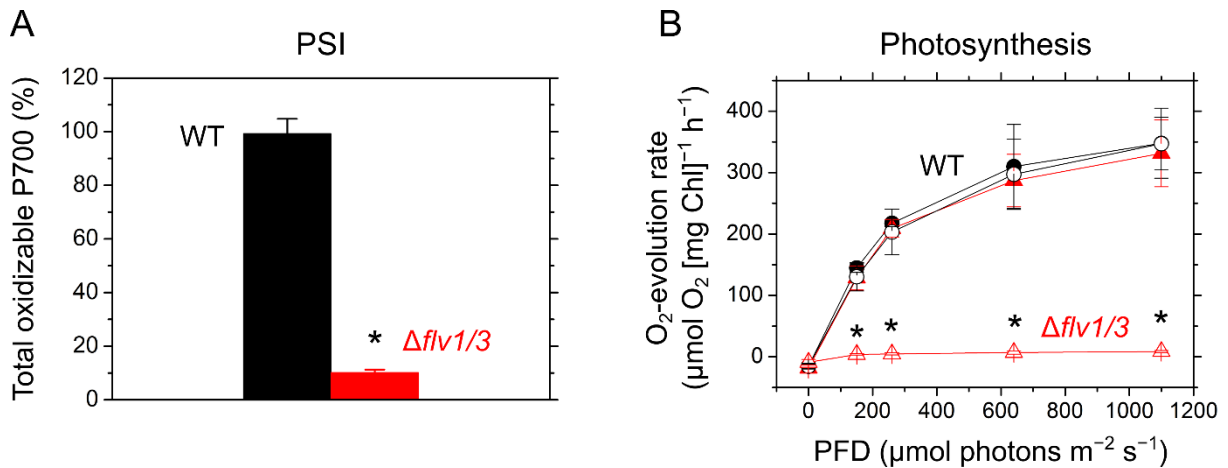


**Fig. 32.** Growth of *S. 7002* WT and the mutant  $\Delta flv1/3$  under ambient  $[\text{CO}_2]$ .  $\text{OD}_{750}$  (A) and Chl (B) measurements were independently conducted three times and the data are shown as means  $\pm$  SD. Black circles, *S. 7002* WT; red triangles,  $\Delta flv1/3$ . Differences between *S. 7002* WT and  $\Delta flv1/3$  were analyzed by Student's *t*-test. Asterisks indicate statistically significant differences between *S. 7002* WT and  $\Delta flv1/3$  at  $*p < 0.05$ .

### Effects of $\text{CO}_2$ limitation on photosynthetic parameters in WT and $\Delta flv1/3$ of *S. 7002*

We hypothesized that PSI photoinhibition occurs under  $\text{CO}_2$  limitation in the absence of FLV-mediated AEF in *S. 7002*. We studied the effects of  $\text{CO}_2$  limitation on total oxidizable P700 (i.e.,  $P_m$ ) and net photosynthetic  $\text{O}_2$  evolution rates in *S. 7002* WT and  $\Delta flv1/3$ . After 2-hour exposure to  $\text{CO}_2$  limitation, neither PSI- nor photosynthesis inactivation was detected in *S. 7002* WT (Fig. 33A and B). Nevertheless, a significant post-treatment reduction in total oxidizable P700 (Fig. 33A) and suppression of photosynthesis (Fig. 33B) were observed in *S. 7002*  $\Delta flv1/3$ .

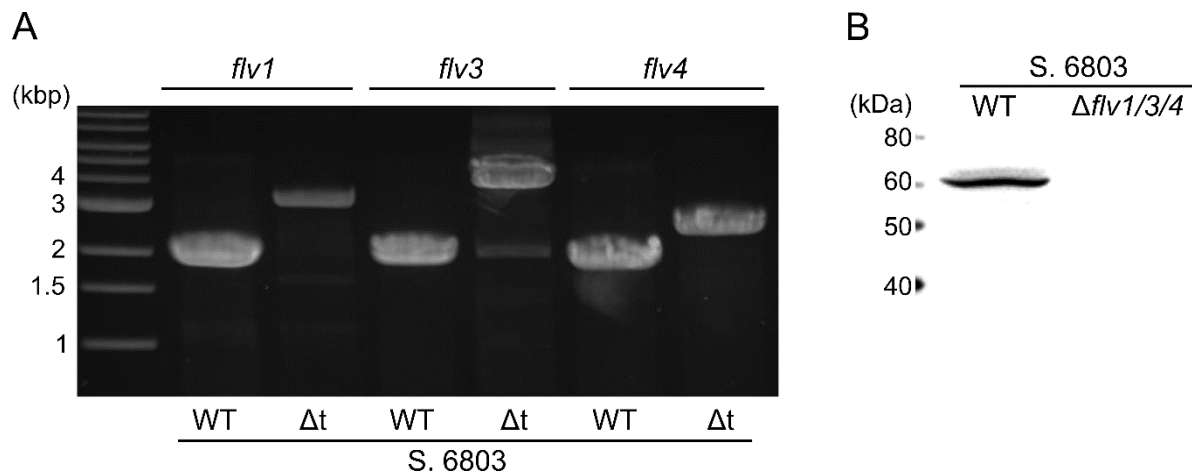
The dramatic decreases in photosynthetic parameters (0–10% of pre-treatment levels; Fig. 33A and B) for  $\Delta flv1/3$  indicate that the lack of FLV1/3 in S. 7002 causes severe PSI photoinhibition under CO<sub>2</sub> limitation.



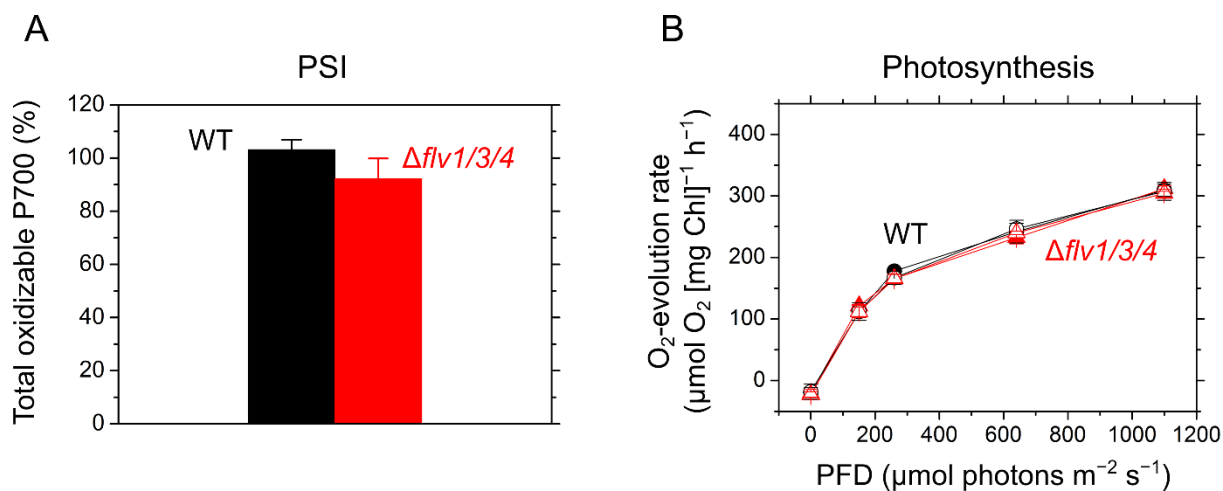
**Fig. 33.** Reduced activities of PSI and photosynthesis in S. 7002 WT and  $\Delta flv1/3$  after 2-hour exposures to CO<sub>2</sub> limitation during illumination (290 μmol photons m<sup>-2</sup> s<sup>-1</sup>). The reaction mixture contained fresh A<sup>+</sup> media and cyanobacterial cells (10 μg Chl mL<sup>-1</sup>). Residual total oxidizable P700 (A) and photosynthetic O<sub>2</sub>-evolution rates (B) were measured before and after 1 h in the dark following treatments. Black and red symbols represent WT and  $\Delta flv1/3$ , respectively. Closed and open symbols represent the data before and after the treatments (B), respectively. Photosynthetic O<sub>2</sub>-evolution rates were measured in the presence of 10 mM NaHCO<sub>3</sub>. Each measurement was conducted three times and means ± SD are shown. Differences between the data before and after the treatments were analyzed by Student's t-test. Asterisks indicate statistically significant differences at \**p* < 0.05.

To determine whether the deletion of FLV-mediated AEF combined with CO<sub>2</sub>

limitation always causes PSI photoinhibition in cyanobacteria, we applied the same treatment to S. 6803 and S. 7942. For S. 6803, we used a mutant deficient in the expression of all four *flv* genes ( $\Delta flv1/3/4$ ) since the WT of this species possesses FLV2/4 (Table 5; Fig. 34) (Eisenhut et al., 2012). Unlike S. 7002, the amounts of total oxidizable P700 were the same before and after the treatment for both S. 6803 and S. 7942 even when the *flv* genes were not expressed (Fig. 35A and 36A). CO<sub>2</sub> limitation also did not affect the dependence of photosynthetic O<sub>2</sub>-evolution rates on PFD in either the WT or the *flv* mutants of S. 6803 and S. 7942 (Fig. 35B and 36B). On the other hand, the deletion of FLV1/3 in S. 7942 decreased photosynthetic O<sub>2</sub> evolution rates even before CO<sub>2</sub> limitation, particularly in high light conditions (Fig. 36B) due to RISE (Shaku et al., 2016).



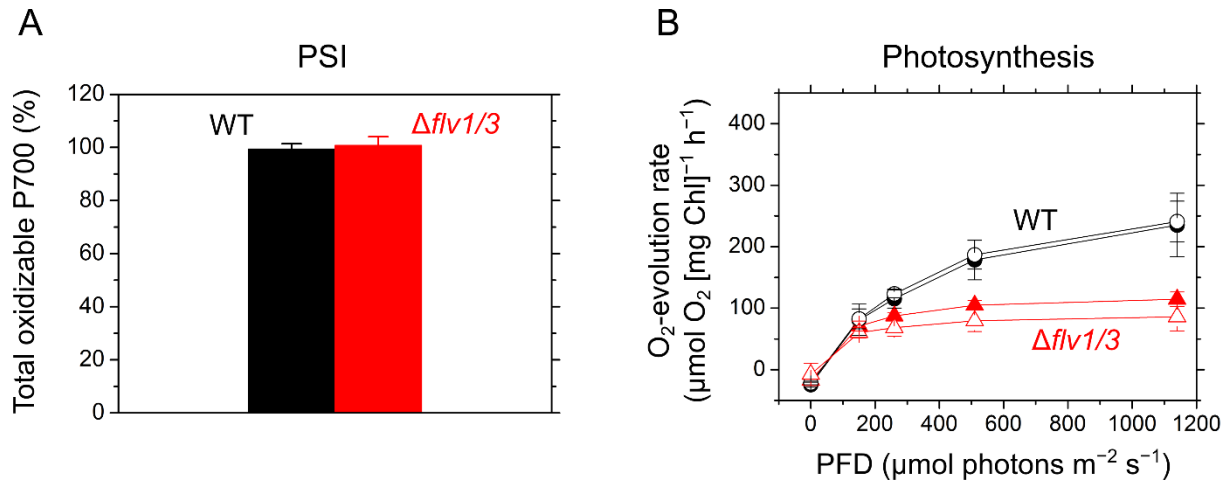
**Fig. 34.** Insertional inactivation of *flv* genes in *S. 6803*. (A) DNA fragments amplified by PCR showing complete segregation of the inactivated genes, *flv1* (*sll1521*) and *flv4* (*sll0217*). WT, *S. 6803* WT;  $\Delta t$ , the triple mutant of *S. 6803* ( $\Delta flv1/3/4$ ). (B) Western blot analysis showing the lack of the *flv3* (*sll0550*) gene product. Extracted soluble fractions (5  $\mu$ g protein/lane) of *S. 6803* WT and  $\Delta flv1/3/4$  were analyzed.



**Fig. 35.** Decreased activities of PSI and photosynthesis in the WT and the *flv* mutants of *S. 6803* after 2-hour exposures to  $CO_2$  limitation during illumination ( $290 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ). Reaction mixtures contained 50 mM HEPES (pH 7.5) and cells ( $10 \mu\text{g Chl mL}^{-1}$ ). Residual total oxidizable P700 (A) and photosynthetic  $O_2$ -evolution rates (B) 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 (B), 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  $\pm$  SD are shown.

### Effects of FLV on the photosynthetic parameters of PSII and PSI in *S. 7002*

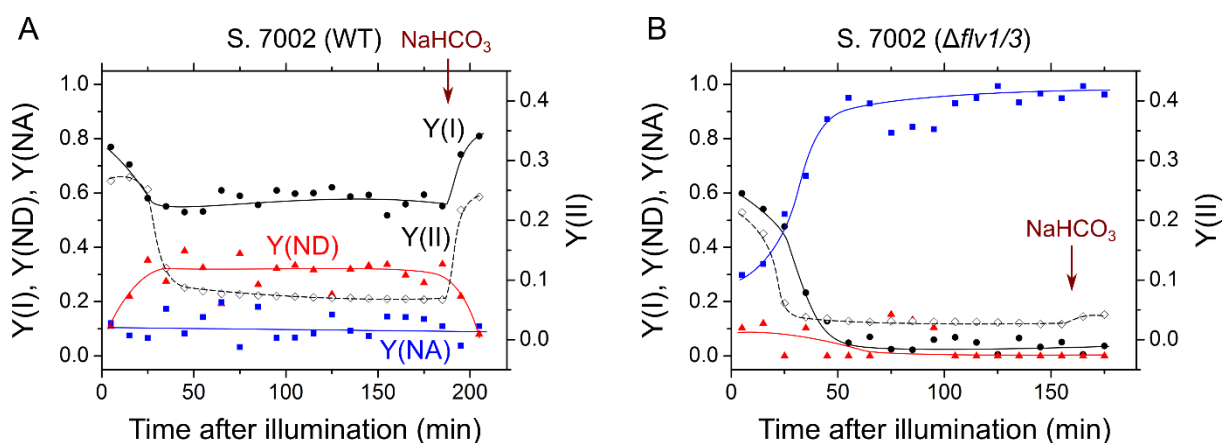
To determine the relationship between PSI photo-damage and P700 oxidation in *S. 7002*, we simultaneously monitored Chl fluorescence and the P700 redox state in PSI during the transition from  $CO_2$  saturation to  $CO_2$  limitation. We modified methods used in our previous work (see



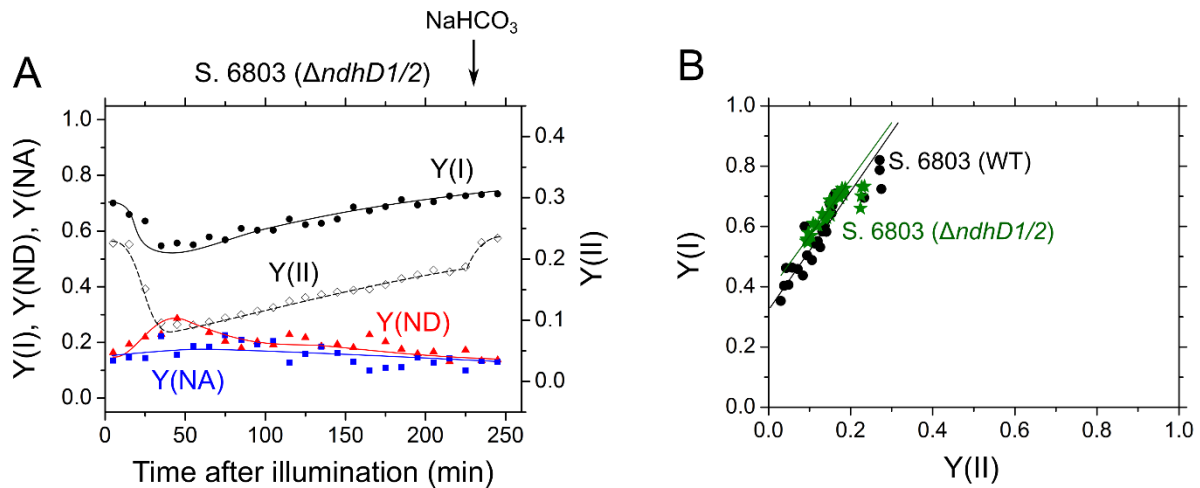
**Fig. 36.** Decreased activities of PSI and photosynthesis in the WT and the *flv* mutants of *S. 7942* after 2-hour exposures to  $CO_2$  limitation during illumination ( $290 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ). Reaction mixtures contained 50 mM HEPES (pH 7.5) and cells ( $10 \mu\text{g Chl mL}^{-1}$ ). Residual total oxidizable P700 (A) and photosynthetic  $O_2$ -evolution rates (B) 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 (B), 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  $\pm$  SD are shown.

the “Part I”) (Hayashi et al., 2014; Shimakawa et al., 2015; Shimakawa et al., 2016). Upon red AL illumination in *S. 7002* WT, incident quantum yields of PSI, i.e., 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. 37A).  $CO_2$  consumption suppressed photosynthesis. Y(I) and Y(II) were restored when  $CO_2$  was added in the form of  $NaHCO_3$  (Fig.

37A) (Hayashi et al., 2014; Shimakawa et al., 2015; Shimakawa et al., 2016). The P700 redox state also responded to CO<sub>2</sub> limitation. The suppression of LEF increased the yield of oxidized P700, i.e., Y(ND). This condition was alleviated by the addition of CO<sub>2</sub> (Fig. 37A). On the other hand, the yield of photo-excited P700, i.e., Y(NA), did not change in response to the shortage of CO<sub>2</sub> (Fig. 37A). Therefore, the PSI acceptor side limitation did not change after *S. 7002* WT was subjected to CO<sub>2</sub> 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 below). The *S. 6803* mutant  $\Delta ndhD1/2$ , which is deficient in the D subunits of NAD(P)H dehydrogenase, however, also had higher Y(I) than Y(II) (Fig. 38). The large gap between Y(I) and Y(II) in cyanobacteria merits further investigation.



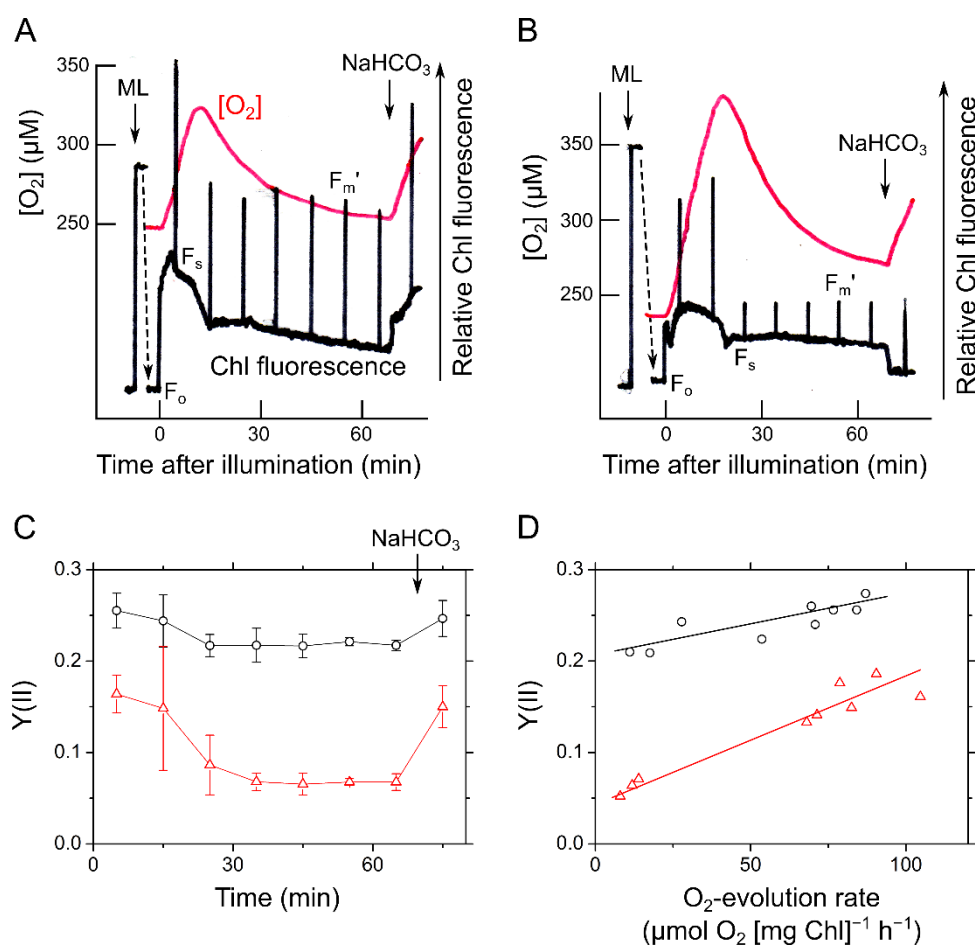
**Fig. 37.** Responses of the photosynthetic parameters of PSII and PSI to CO<sub>2</sub> limitation in *S. 7002* WT (A) and the mutant  $\Delta flv1/3$  (B). Reaction mixtures contained the cells (10  $\mu$ g Chl mL<sup>-1</sup>). Y(I), black circles; Y(ND), red triangles; Y(NA), blue squares; Y(II), white diamonds. Red AL (180  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>) was activated at time zero. NaHCO<sub>3</sub> (10 mM) was added as indicated. Measurements were conducted three times, and representative data are shown.



**Fig. 38.** Responses of the photosynthetic parameters of PSII and PSI to CO<sub>2</sub> limitation in the mutant of *S. 6803* deficient in *ndhD1* and 2 ( $\Delta ndhD1/2$ ). Reaction mixtures contained the cells ( $10 \mu\text{g Chl mL}^{-1}$ ). (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 ( $180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was activated at time zero.  $\text{NaHCO}_3$  ( $10 \text{ mM}$ ) was added as indicated. Measurements were conducted three times, and representative data are shown. The data of *S. 6803* WT is shown in Fig. 40. (B) The relationship between  $Y(II)$  and  $Y(I)$  throughout the measurement in *S. 6803* WT (Black circles) and  $\Delta ndhD1/2$  (green stars). Data plotted are obtained from Fig. 38A and 40A, respectively.

Next, we measured the photosynthetic parameters of PSII and PSI after the transition to CO<sub>2</sub>-limitation in  $\Delta flv1/3$  of *S. 7002*. Before CO<sub>2</sub> deprivation,  $Y(I)$  and  $Y(II)$  in  $\Delta flv1/3$  were lower (about 0.6 and 0.2, respectively) than those in WT, whereas  $Y(NA)$  was higher in the mutant (about 0.3) than the WT (about 0.1) (Fig. 37). These results imply that FLV1/3 drives AEF in *S. 7002*, as it does for *S. 6803* (Helman et al., 2003; Helman et al., 2005) and *S. 7942* (Shaku et al., 2016). CO<sub>2</sub> limitation did not induce P700 oxidation in *S. 7002*  $\Delta flv1/3$  (Fig. 37B).

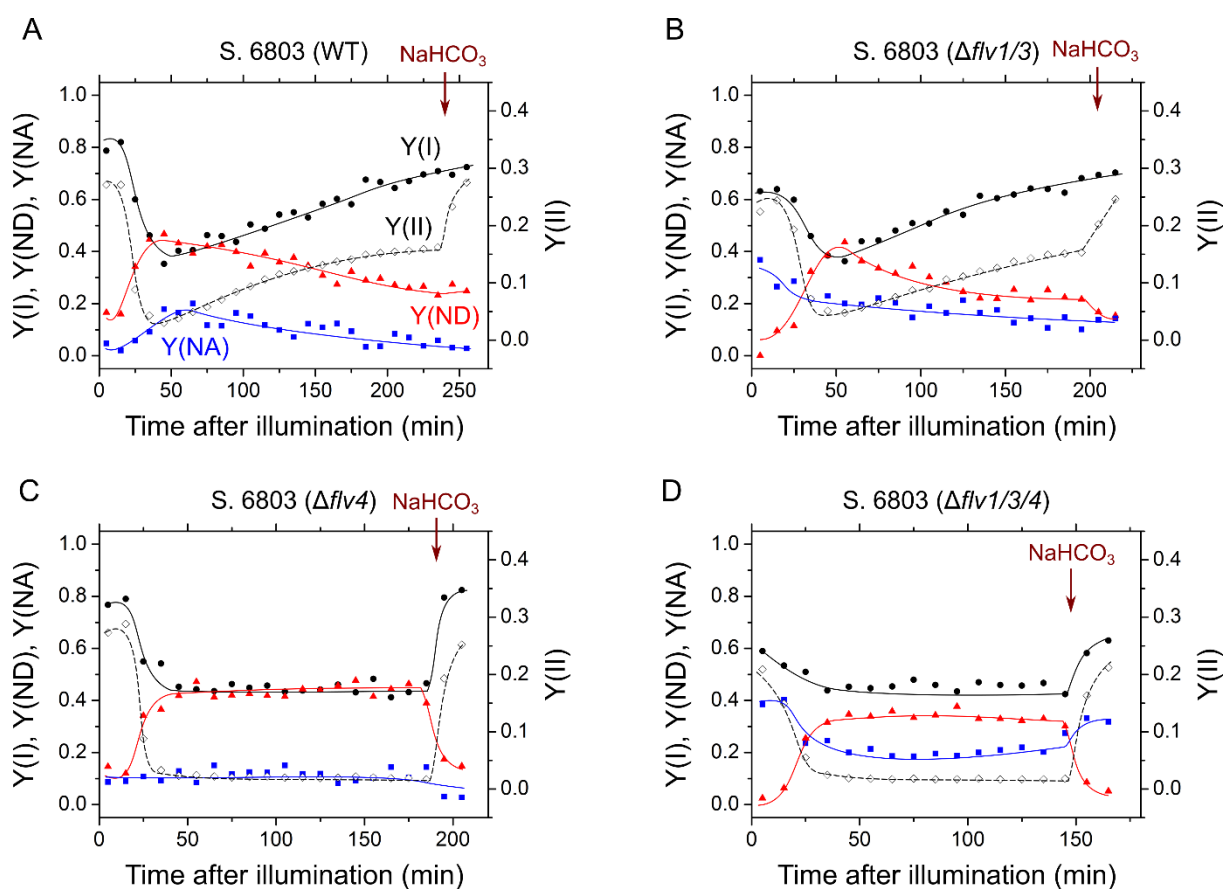
An increase in  $Y(\text{NA})$  indicated that the electron flux from P700 to the acceptor side of PSI was reduced further still (Fig. 37B).  $Y(\text{I})$  was also considerably suppressed under  $\text{CO}_2$  limitation (Fig. 37B). The addition of  $\text{NaHCO}_3$  did not restore  $Y(\text{I})$  or  $Y(\text{II})$  (Fig. 37B). These results suggest that unless FLV1/3-mediated AEF is active, PSI photoinhibition occurs in S. 7002 during  $\text{CO}_2$  limitation. For S. 7002, FLV1/3 plays a primary role in oxidizing the PET system under  $\text{CO}_2$  limitation. Recently, we found that S. 7002 drives an  $\text{O}_2$ -dependent AEF to restore linear electron transport during  $\text{CO}_2$ -limited photosynthesis. This process is particularly evident in cells grown under ambient  $[\text{CO}_2]$  (Shimakawa et al., 2016). In the present study, a simultaneous measurement of  $[\text{O}_2]$  and Chl fluorescence was performed in  $\Delta flv1/3$  of S. 7002 grown under ambient  $[\text{CO}_2]$  (Fig. 39), indicating that FLV1/3 is the molecular mechanism of the  $\text{O}_2$ -dependent AEF we found in S. 7002 (Shimakawa et al., 2016).



**Fig. 39.** *Photosynthetic parameters of S. 7002 WT and  $\Delta flv1/3$  grown in ambient  $[CO_2]$ . (A, B) Time courses of dissolved  $O_2$  concentration (red lines) and relative Chl fluorescence yield (black lines) in S. 7002 WT (A) and  $\Delta flv1/3$  (B). Experimental procedures were similar to those in our recent study (Shimakawa et al., 2016). Cells ( $10 \mu g$  Chl  $mL^{-1}$ ) were illuminated with ML as indicated. Red AL ( $300 \mu mol$  photons  $m^{-2} s^{-1}$ ,  $620 < \lambda < 695$  nm) was activated at time zero. Dashed arrows indicate changes in the position of the Chl fluorescence signal. Chl fluorescence parameters are shown as in the figures in the “Part I” and “Part II”.  $NaHCO_3$  (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  $\pm$  SD ( $n = 3$ ). Black circles, S. 7002 WT; red triangles,  $\Delta flv1/3$ . (D) Relationships between gross  $O_2$  evolution rates and  $Y(II)$  in S. 7002 WT (black circles) and  $\Delta flv1/3$  (red triangles). Photosynthetic  $O_2$  evolution rates were determined at both  $CO_2$ -saturated and  $CO_2$ -limited conditions in separate experiments ( $n = 3$ ) following the methods described in the “Part I”.*

### **Effects of FLV on the photosynthetic parameters of PSII and PSI in S. 6803 and S. 7942**

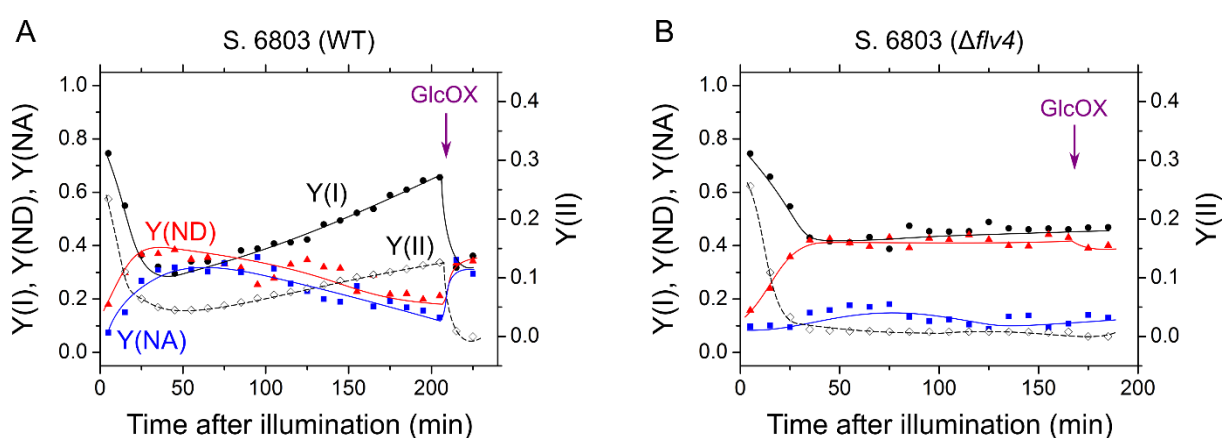
The photosynthetic parameters of PSII and PSI responded differently to  $CO_2$  limitation in S. 6803 than they did in S. 7002. In S. 6803 WT,  $Y(I)$  and  $Y(II)$  decreased to minimum values then started to recover, reaching approximately 90% and 60% of the initial values respectively without the addition of  $NaHCO_3$  (Fig. 40A). This recovery occurred due to the activation of an  $O_2$ -dependent AEF driven by FLV2/4 but not by FLV1/3 (Hayashi et al., 2014; Shimakawa et al., 2015) (Fig. 40C and 41). The AEF stimulated linear electron flow, which decreased both  $Y(ND)$  and  $Y(NA)$  (Fig. 40A). In S. 6803, the deletion of FLV1/3 reduced both  $Y(I)$  and  $Y(II)$



**Fig. 40.** Responses of the photosynthetic parameters of PSII and PSI to  $\text{CO}_2$  limitation in *S. 6803* WT (A) and the mutants  $\Delta flv1/3$  (B),  $\Delta flv4$  (C) and  $\Delta flv1/3/4$  (D). Reaction mixtures contained the cells ( $10 \mu\text{g Chl mL}^{-1}$ ).  $Y(I)$ , black circles;  $Y(ND)$ , red triangles;  $Y(NA)$ , blue squares;  $Y(II)$ , white diamonds. Red AL ( $180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was activated at time zero.  $\text{NaHCO}_3$  ( $10 \text{ mM}$ ) was added as indicated. Measurements were conducted three times and representative data are shown.

relative to the WT before  $\text{CO}_2$  consumption (Fig. 40B). For  $\Delta flv1/3$  of *S. 6803* before  $\text{CO}_2$  depletion,  $Y(ND)$  was lower than that of the *S. 6803* WT, whereas the  $Y(NA)$  in the mutant was higher than that of the WT (Fig. 40B). These findings concur with those of previous studies

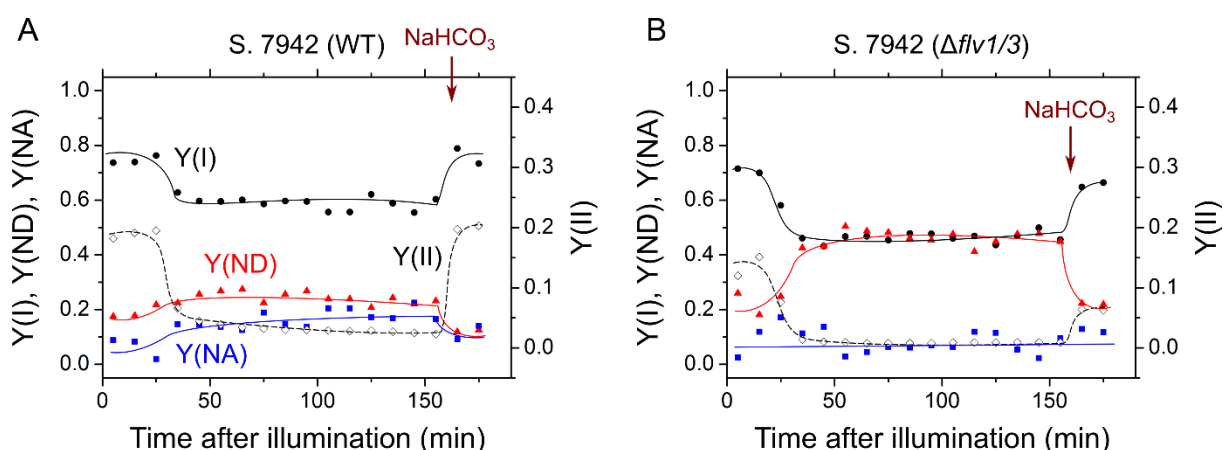
showing that FLV1/3-mediated AEF can oxidize P700 (Helman et al., 2003; Helman et al., 2005; Allahverdiyeva et al., 2011; Allahverdiyeva et al., 2013; Hayashi et al., 2014). The suppression of photosynthetic linear electron flow caused by CO<sub>2</sub> limitation induced P700 oxidation in *S. 6803* even in the absence of FLV-mediated electron flow (Fig. 40D). The oxidized P700 was reduced by the activation of FLV2/4-mediated AEF or by the resumption of photosynthetic CO<sub>2</sub> fixation (Fig. 40).



**Fig. 41.** Effects of eliminating O<sub>2</sub> on the photosynthetic parameters of PSII and PSI under CO<sub>2</sub> limitation in *S. 6803* WT (A) and the mutant  $\Delta flv4$  (B). Reaction mixtures contained the cells (10  $\mu\text{g Chl mL}^{-1}$ ). Y(I), black circles; Y(ND), red triangles; Y(NA), blue squares; Y(II), white diamonds. Red AL (180  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was activated at time zero. Glucose (5 mM), catalase (250 units  $\text{mL}^{-1}$ ), and glucose oxidase (GlcOX, 5 units  $\text{mL}^{-1}$ ) were added as indicated. Measurements were conducted three times and representative data are shown.

*S. 7942* WT lacks FLV2/4-mediated AEF (Hayashi et al., 2014; Shimakawa et al., 2015; 嶋川銀河, 2015), so its PSII and PSI photosynthetic parameters responded to CO<sub>2</sub> limitation in almost the same manner as did those of  $\Delta flv4$  of *S. 6803*. For the *S. 7942* WT, both

Y(I) and Y(II) decreased and remained low under CO<sub>2</sub> limitation, but were restored by adding NaHCO<sub>3</sub> (Fig. 42A). The increase in Y(ND) reflected P700 oxidation in response to CO<sub>2</sub> limitation and was observed in both the WT and  $\Delta flv1/3$  of *S. 7942* (Fig. 42). The mutant of *S. 7942* also had a higher Y(ND) than did the *S. 7942* WT under CO<sub>2</sub> limitation (Fig. 42B). These results align with the findings of a previous study (Shaku et al., 2016).



**Fig. 42.** Responses of the photosynthetic parameters of PSII and PSI to CO<sub>2</sub> limitation in *S. 7942* WT (A) and the mutant  $\Delta flv1/3$  (B). Reaction mixtures contained the cells (10  $\mu\text{g Chl mL}^{-1}$ ). Y(I), black circles; Y(ND), red triangles; Y(NA), blue squares; Y(II), white diamonds. Red AL (180  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was activated at time zero. NaHCO<sub>3</sub> (10 mM) was added as indicated. Measurements were conducted three times and representative data are shown.

## 6.4. Discussion

**Table 7.** Phenotypes of each WT and *flv* mutant under CO<sub>2</sub> limitation in three cyanobacteria species used in the “Part III”

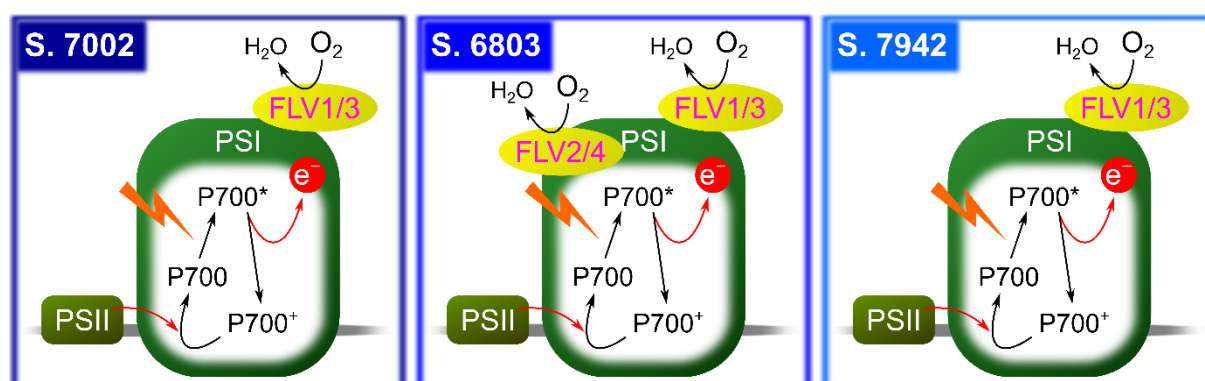
|         |                   | Growth             | Y(II)              | Y(I)               | P700 redox             | PSI                |
|---------|-------------------|--------------------|--------------------|--------------------|------------------------|--------------------|
| S. 7002 | WT                | Well               | High <sup>*1</sup> | High <sup>*1</sup> | Oxidized               | Safe               |
|         | $\Delta flv1/3$   | Bad                | Low                | Low                | <b>Reduced</b>         | <b>Broken</b>      |
| S. 6803 | WT                | Well               | High <sup>*2</sup> | High               | Oxidized               | Safe               |
|         | $\Delta flv1/3$   | Well <sup>*3</sup> | High <sup>*2</sup> | High               | Oxidized               | Safe               |
|         | $\Delta flv4$     | Bad <sup>*3</sup>  | Low <sup>*4</sup>  | Low                | Oxidized               | Safe <sup>*3</sup> |
|         | $\Delta flv1/3/4$ | –                  | Low                | Low                | Oxidized               | Safe               |
| S. 7942 | WT                | Well               | Low <sup>*2</sup>  | Low <sup>*5</sup>  | Oxidized <sup>*5</sup> | Safe <sup>*5</sup> |
|         | $\Delta flv1/3$   | Well <sup>*5</sup> | Low <sup>*5</sup>  | Low <sup>*5</sup>  | Oxidized <sup>*5</sup> | Safe <sup>*5</sup> |

<sup>\*1</sup>Shimakawa et al. (2016); <sup>\*2</sup>Hayashi et al. (2014); <sup>\*3</sup>Zhang et al. (2009); <sup>\*4</sup>Shimakawa et al. (2015);

<sup>\*5</sup>Shaku et al. (2016).

Table 7 summarizes the findings of previous and current studies, and shows two main conclusions: (a) P700 oxidation is directly linked to the protection of PSI against photoinhibition, and (b) in cyanobacteria, there are several strategies, including FLV, to alleviate PSI photoinhibition. In S. 7002, FLV1/3 mediates O<sub>2</sub>-dependent AEF that regulates the PSI redox state and promotes P700 oxidation under CO<sub>2</sub> limitation (Fig. 37 and 39; see the “Part I”). In S. 7002, the lack of FLV-mediated AEF resulted in P700 reduction, photosynthesis suppression, PSI photoinhibition, and growth retardation (Fig. 32 and 33). These observations

correspond to higher transcript levels of *flv1/3* under CO<sub>2</sub> limitation (Ludwig and Bryant, 2011; Ludwig and Bryant, 2012, 2012). In contrast, *S. 6803* and *S. 7942* keep P700 oxidized under CO<sub>2</sub> limitation independently of FLV-mediated AEF (which protects PSI against photo-oxidative damage). In cyanobacteria, FLV has diverse physiological significances as the agent for O<sub>2</sub>-dependent AEF (Fig. 43).



**Fig. 43.** A summary diagram of diverse strategies to oxidize P700 under CO<sub>2</sub> limitation for the protection of PSI against the photo-oxidative damage derived from ROS among three cyanobacteria species, including *S. 7002*, *S. 6803*, and *S. 7942*.

In the present study, we showed that P700 oxidation protects PSI against photoinhibition in cyanobacteria (which are the progenitors of oxygenic phototrophs). Photo-oxidative damage in PSI is caused by ROS generated by excitation energy transfer from P700 ultimately to O<sub>2</sub>. Therefore, photo-oxidizable P700 in PSI can produce ROS, whereas oxidized P700 cannot be excited by photon energy. PSI photoinhibition is caused by O<sub>2</sub><sup>-</sup> produced on the acceptor side of PSI when NADP<sup>+</sup> regeneration is limited (Hihara and Sonoike, 2001). Added hydrogen peroxide reacts with reduced iron in iron-sulfur centers to form ·OH that

destroy PSI instantaneously (Sonoike, 1996; Sonoike et al., 1997; Sonoike, 2011). P700 oxidation is expected to negate the effect of  $\cdot\text{OH}$  by suppressing  $\text{O}_2^-$  generation and by oxidizing the iron-sulfur centers (Sonoike et al., 1995; Sonoike, 1996). Recently, it was suggested that  $^1\text{O}_2$  triggers PSI photoinhibition (Cazzaniga et al., 2012; Cazzaniga et al., 2016; Takagi et al., 2017). Keeping P700 oxidized should help suppress  $^1\text{O}_2$  generation. P700 oxidation alleviates PSI photoinhibition in sunflower 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 photo-oxidative damage. P700 oxidation would be a hedge against ROS generation.

In S. 6803 and S. 7942, P700 remained oxidized under  $\text{CO}_2$  limitation even without FLV1/3 and FLV2/4 (Fig. 40 and 42). There is, therefore, a P700 oxidation mechanism that operates independently of FLV-mediated AEF under  $\text{CO}_2$  limitation. One candidate is CEF around PSI, which helps induce  $\Delta\text{pH}$  across thylakoid membrane (Endo et al., 1998; Miyake et al., 2004; Miyake et al., 2005). Acidification of the lumenal side reduces the oxidation activity of  $\text{PQH}_2$  in the Cyt  $b_6/f$ -complex and limits the electron flux from  $\text{PQH}_2$  to P700 through plastocyanin (or Cyt  $c$ ). These hypotheses are supported by the fact that  $Y(\text{I}) > Y(\text{II})$  for all cyanobacterial strains tested except for the  $\Delta flv1/3$  of S. 7002 (Fig. 40 and 42). Nevertheless, we also found that  $Y(\text{I}) > Y(\text{II})$  both for S. 6803 WT and its mutant  $\Delta ndhD1/2$  (Fig. 38). Therefore, reduced activity of NDH-mediated CEF (Ohkawa et al., 2000; Ohkawa et al., 2000) is not linked to  $Y(\text{I}):Y(\text{II})$ . Moreover, the electron transport rates of CEF in S. 6803 and S. 7002 are negligible relative to their photosynthetic linear and respiratory electron transport rates (Yu et al., 1993; Shimakawa et al., 2015) (Fig. 16, see the “Part II”). We could not determine why  $Y(\text{I})$  differed from  $Y(\text{II})$  in cyanobacteria. The contribution of phycobiliprotein to  $F_0$  fluorescence may account for it (Campbell et al., 1998), as might the difference between the quality of the growth light and that of the LED-sourced AL used in the experiments. Another

candidate for the P700 oxidation mechanism is the suppression of the Q-cycle when PQ is reduced. In this case, the electron flux from Cyt *b6/f*-complex to P700 in PSI decreases. This response is called RISE (Shaku et al., 2016; 釋啓一郎 and 三宅親弘, 2016) and it might be the main driver of P700 oxidation under CO<sub>2</sub> limitation in cyanobacteria. Respiratory terminal oxidases (i.e., RTOs) like Cyt *c* oxidase and *bd*-type quinol oxidase may also contribute to the oxidation of the donor side of PSI under CO<sub>2</sub> limitation (Beardall et al., 2003; Lea-Smith et al., 2013; Lea-Smith et al., 2014; Ermakova et al., 2016). It is, however, difficult to explain why Y(II) decreased during CO<sub>2</sub> limitation in the cyanobacteria we studied (Fig. 37, 40, and 42).

In *S. 6803*, FLV2/4 may receive electrons from the acceptor side of PSI. In both *S. 6803* WT and its mutant  $\Delta flv1/3$ , the increases in Y(I) and Y(II) indicate that PSI electron flux is restored under CO<sub>2</sub> limitation (Fig. 40 and 41). Unlike *S. 6803* WT and  $\Delta flv1/3$ , neither  $\Delta flv4$  nor  $\Delta flv1/3/4$  experienced an increase in Y(I) (Fig. 40C and D). Removing O<sub>2</sub> lowered Y(I) under CO<sub>2</sub> limitation in *S. 6803* WT but not in  $\Delta flv4$  (Fig. 41). These data suggest that FLV2/4 mediates an O<sub>2</sub>-dependent AEF on the acceptor side of PSI (Hayashi et al., 2014; Shimakawa et al., 2015) as does FLV1/3 (Helman et al., 2003; Helman et al., 2005) since both FLV sets have similar primary structures (Fujisawa et al., 2014; Fujisawa et al., 2017) and enzymatic characteristics of recombinant proteins (Vicente et al., 2002; Vicente et al., 2008; Shimakawa et al., 2015). However, we cannot exclude the possibility that the relief of excitation pressure at PSII by FLV2/4 (Bersanini et al., 2014) provides an enhancement of Y(I) during CO<sub>2</sub>-limited photosynthesis in *S. 6803*. FLV2/4 is known to interact with PSII and phycobilisomes (Bersanini et al., 2014; Bersanini et al., 2017), so in *S. 6803* it may have multiple functions to alleviate photoinhibition under low CO<sub>2</sub>.

## Part IV

The liverwort, *Marchantia*, drives alternative electron flow using a flavodiiron protein to protect PSI

This part is based on the following publication:

**Ginga Shimakawa, Kimitsune Ishizaki, Shigeyuki Tsukamoto, Moeko Tanaka, Takehiro Sejima, Chikahiro Miyake** (2017) The liverwort, *Marchantia*, drives alternative electron flow using a flavodiiron protein to protect PSI. *Plant Physiology* 173(3) 1636–1647.

## 7.1. Abstract

The diffusion efficiency of O<sub>2</sub> in atmosphere, like that of CO<sub>2</sub>, is approximately 10<sup>4</sup> times greater than that in aqueous environments. Consequently, terrestrial photosynthetic organisms need mechanisms to protect against potential oxidative damage. The liverwort *Marchantia polymorpha*, a basal land plant, has habitats where it is exposed to both water and the atmosphere. Furthermore, like cyanobacteria, *M. polymorpha* has genes encoding FLV. In cyanobacteria, FLVs mediate O<sub>2</sub>-dependent AEF to suppress production of ROS. Here, we investigated whether FLVs are required for the protection of photosynthesis in *M. polymorpha*. A mutant deficient in the FLV1 isozyme ( $\Delta MpFlv1$ ) sustained photo-oxidative damage to PSI following repetitive short-saturation pulses of light. Compared with the wild type (Tak-1),  $\Delta MpFlv1$  showed the same photosynthetic O<sub>2</sub>-evolution rate, but a lower electron transport rate during the induction phase of photosynthesis. Additionally, the reaction center Chl in PSI, P700, was highly reduced in  $\Delta MpFlv1$ , but not in Tak-1. These results indicate that the gene product of *MpFlv1* drives AEF to oxidize PSI, as in cyanobacteria. Further, FLV-mediated AEF supports the production of a proton motive force to possibly induce non-photochemical quenching of Chl fluorescence and suppress electron transport in Cyt *b<sub>6</sub>/f*. After submerging the thalli, a decrease in Y(II) was observed, particularly in  $\Delta MpFlv1$ , which implies that species living in these sorts of habitats require FLV-mediated AEF.

## 7.2. Introduction

A decrease in the efficiency of light usage for photosynthetic CO<sub>2</sub>-fixation in the Calvin-Benson cycle enhances the likelihood that oxygenic phototrophs will suffer from photo-oxidative damage caused by ROS (Asada, 2000; Asada, 2006). For example, under conditions of high light, low CO<sub>2</sub>, and/or low temperature, light energy absorbed by PSI and PSII in thylakoid membranes exceeds requirements, because turnover of the Calvin-Benson cycle limits the regeneration of NADP<sup>+</sup>. Consequently, electrons accumulate in the photosynthetic electron transport system. The accumulated electrons start to flow to O<sub>2</sub>, producing ROS, including O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>, in PSI (Bolwell and Wojtaszek, 1997; Apel and Hirt, 2004; Krieger-Liszkay, 2005; Murata et al., 2007; Krieger-Liszkay et al., 2011; Sejima et al., 2014; Dietz et al., 2016; Takagi et al., 2016). Furthermore, the accumulation of electrons in the photosynthetic electron transport system suppresses charge separation of the reaction center Chl, P680 in PSII and P700 in PSI, which stimulates the production of another ROS, <sup>1</sup>O<sub>2</sub> (Cazzaniga et al., 2012; Fischer et al., 2013; Cazzaniga et al., 2016). It is proposed that the reduced state of P700 in PSI under illuminated conditions stimulates the production of <sup>1</sup>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> to inactivate PSI, resulting in a decline in photosynthetic CO<sub>2</sub> fixation (Cazzaniga et al., 2012; Rutherford et al., 2012; Sejima et al., 2014; Zivcak et al., 2015; Zivcak et al., 2015; Cazzaniga et al., 2016; Takagi et al., 2016; Takagi et al., 2017).

Oxygenic phototrophs have various systems that can be used to suppress electron accumulation in the photosynthetic electron transport system; they are broadly divided into two groups. The first group uses regulation of the absorption efficiency of photon energy into the photosynthetic electron transport system, which includes NPQ and state transition (Ruban et al., 2012; Niyogi and Truong, 2013; Kromdijk et al., 2016; Ruban, 2016). In the second group, electron sinks consume excess photon energy as futile electron pathways with heat dissipation.

These electron sinks constitute AEF, and the representatives are observed as photorespiration and Mehler-like reactions (Kozaki and Takeba, 1996; Wiese et al., 1998; Helman et al., 2003; Helman et al., 2005; Laisk et al., 2007; Miyake, 2010; Allahverdiyeva et al., 2011; Allahverdiyeva et al., 2013; Bersanini et al., 2014; Hayashi et al., 2014; Allahverdiyeva et al., 2015; Shimakawa et al., 2015; Sejima et al., 2016; Shimakawa et al., 2016; Takagi et al., 2016; Hanawa et al., 2017).

With regard to the protection of PSI against photo-oxidative damage, there is a further strategy in addition to NPQ and AEF that suppresses photosynthetic electron transport in Cyt *b<sub>6</sub>/f* in the center of the photosynthetic electron transport system. This suppression is triggered by the integration of ( $\Delta pH$ ) across thylakoid membranes (Stiehl and Witt, 1969; Witt, 1979; Tikhonov et al., 1981; Kramer et al., 1999; Kramer et al., 2003; Kramer et al., 2004; Tikhonov, 2012; Tikhonov, 2013, 2014) or the reduction of the PQ pool (Shaku et al., 2016), which contributes to the oxidation of PSI. Furthermore,  $\Delta pH$  is also involved in the induction of the molecular mechanisms of some NPQ (Schöttler and Tóth, 2014; Chaux et al., 2015; Schöttler et al., 2015). Collectively, oxygenic phototrophs harbor many systems for the alleviation of photo-oxidative damage, which are rich in diversity and have been acquired or lost by species over their evolutionary history.

One of the most enduring mysteries is whether a molecular mechanism of AEF has been lost over the long history of evolutionary processes in oxygenic phototrophs. In the progenitor cyanobacteria, Kaplan's research group found a large AEF to O<sub>2</sub>, which is not photorespiration, and showed that the AEF is driven by FLV1/3 (Helman et al., 2003; Helman et al., 2005). Some cyanobacteria have four isozymes of FLV (FLV1–4), whereas others have just two: FLV1 and 3. FLV1/3 and FLV2/4 function, respectively, as heterodimers (Zhang et al., 2012; Allahverdiyeva et al., 2013). In *S. 6803*, both FLV1/3 and FLV2/4 mediate O<sub>2</sub>-dependent AEF, as well as oxidize the photosynthetic electron transport system under CO<sub>2</sub>-saturated and

CO<sub>2</sub>-limited conditions, respectively (Helman et al., 2003; Hayashi et al., 2014; Shimakawa et al., 2015). A S. 6803 mutant deficient in FLV1/3 was shown to sustain oxidative damage under fluctuating light (Allahverdiyeva et al., 2013; Allahverdiyeva et al., 2015), and another mutant deficient in FLV2/4 also suffered under low CO<sub>2</sub> conditions (Zhang et al., 2009; Zhang et al., 2012). Furthermore, a S. 7002 mutant deficient in FLV1/3 suffers from PSI photoinhibition under CO<sub>2</sub>-limited conditions (see the “Part III”) (Shimakawa et al., 2016). These data indicate that FLV is essential for the alleviation of photo-oxidative damage in cyanobacteria. Surprisingly, during the course of evolution, the isozymes of FLV have been lost in seed plants at the gene level (Zhang et al., 2009; Allahverdiyeva et al., 2015; Yamamoto et al., 2016). On the basis of accumulated genomic information, FLV1/3 isozymes are found to be broadly conserved in the photosynthetic green plant lineage, including cyanobacteria, green algae, bryophytes, pteridophytes, and pinophytes; there is more sequence variation between basal land plants and cyanobacteria than there is among cyanobacteria (Yamamoto et al., 2016). This suggests that at some point in the course of evolution, oxygenic phototrophs no longer had a need for FLV. However, the physiological functions of FLV in phototrophs other than cyanobacteria have yet to be elucidated.

The liverwort *Marchantia polymorpha* is positioned between algae and plants in the photosynthetic green plant lineage (Bowman et al.; Bowman et al., 2007), and has two genes encoding isozymes of FLV1/3 (*MpFlv1*, *Mapoly0005s0210*; *MpFLV3*, *Mapoly0103s0039*). Additionally, one of the habitats of *M. polymorpha* is the marginal area between aquatic and land environments; thus, this plant sometimes experiences both submergences and drought stress. Submergence limits the diffusion of gases. The diffusion coefficient of CO<sub>2</sub> in water is approximately 10<sup>-4</sup> times lower than that in the atmosphere, which depresses photosynthetic CO<sub>2</sub>-fixation (Van et al., 1976; Armstrong, 1980; Raven et al., 1985; Badger and Spalding, 2000; Raven et al., 2014). Furthermore, another habitat of the liverwort is the forest floor, where

photon energy from the sun is provided as fluctuating light termed “sunfleck” (Boardman, 1977). These habitat situations suggest that *M. polymorpha* would require sufficient AEF activity to escape from oxidative damage through the dissipation of excess photon energy.

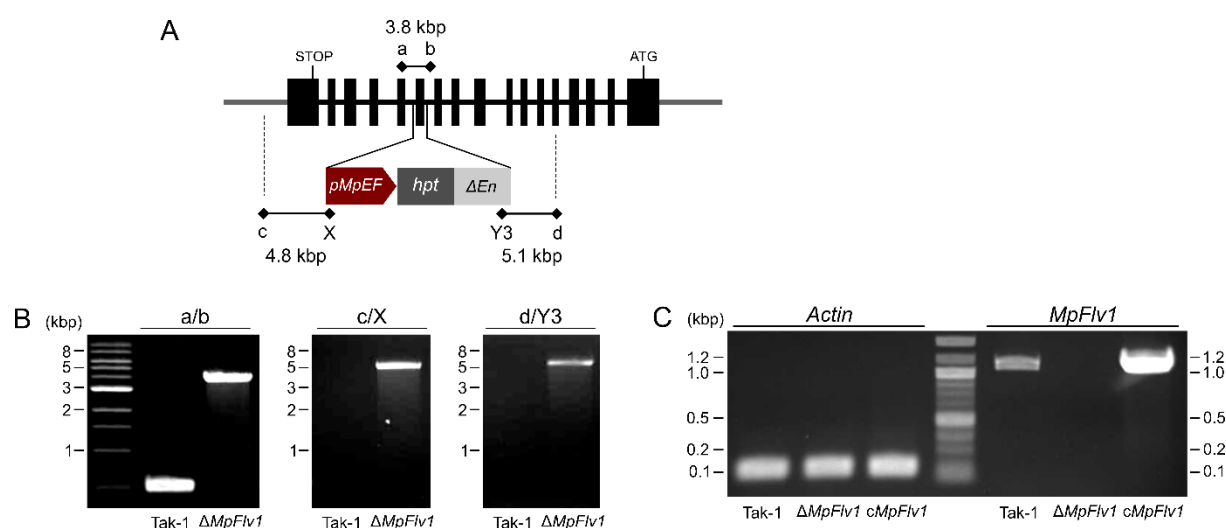
In the present study, we sought to clarify the physiological functions of FLV in *M. polymorpha* by comparing the wild type (hereafter Tak-1) and a mutant deficient in *MpFlv1* ( $\Delta MpFlv1$ ). We found that the activity of AEF was mediated by FLV, which supports the formation of  $\Delta pH$  that contributes to the protection of PSI against photo-oxidative damage under the fluctuating light conditions in which *M. polymorpha* grows.

### 7.3. Results and Discussion

**Table 8.** Characteristics of Tak-1,  $\Delta MpFlv1$ , and  $cMpFlv1$

|                 | $F_v/F_m$         | Total Chl ( $\text{g m}^{-2}$ ) | Chl <i>a</i> / Chl <i>b</i> | Total N ( $\text{g m}^{-2}$ ) |
|-----------------|-------------------|---------------------------------|-----------------------------|-------------------------------|
| Tak-1           | $0.811 \pm 0.008$ | $0.20 \pm 0.03$                 | $2.97 \pm 0.12$             | $0.79 \pm 0.12$               |
| $\Delta MpFlv1$ | $0.804 \pm 0.004$ | $0.20 \pm 0.01$                 | $3.08 \pm 0.04$             | $0.76 \pm 0.06$               |
| $cMpFlv1$       | $0.803 \pm 0.018$ | $0.22 \pm 0.04$                 | $3.03 \pm 0.08$             | $0.73 \pm 0.11$               |

Data are shown as mean  $\pm$  SD of three independent measurements.



**Fig. 44.** Strategy for targeted disruption of the *MpFlv1* locus and analysis of homologous recombination events. (A) Schematic diagram of targeted disruption of the *MpFlv1* locus through homologous recombination. (B) Genotyping of the  $\Delta MpFlv1$  lines. The positions of the primers used for PCR analysis are shown in Fig. 44A. (C) Reverse transcript-PCR of *MpFlv1* in Tak-1,  $\Delta MpFlv1$ , and  $cMpFlv1$ .



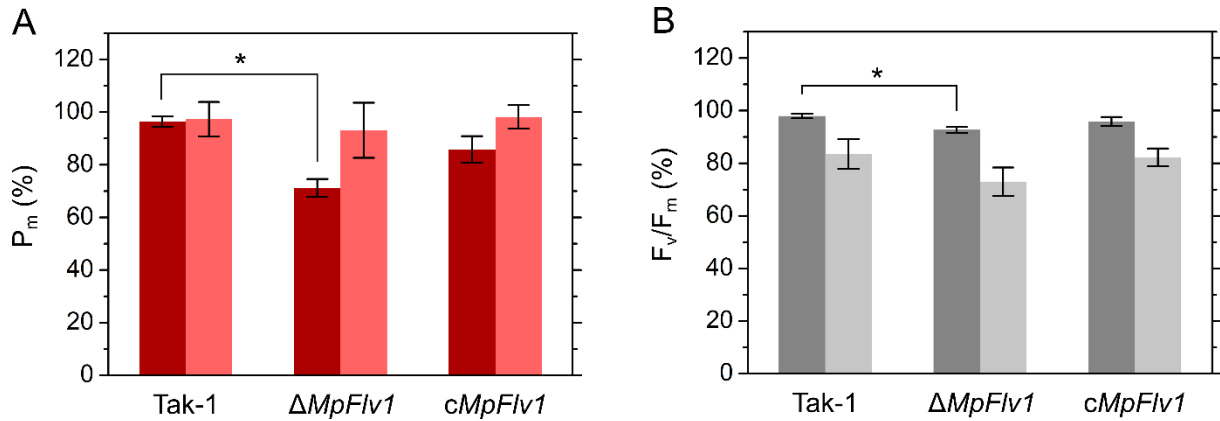
**Fig. 45.** A photo of the thalli of Tak-1,  $\Delta MpFlv1$ , and  $cMpFlv1$  used in this study. A white bar indicates 1 cm.

### Effects of FLV on photo-oxidative damage to PSI in *M. polymorpha*

We constructed the mutant  $\Delta MpFlv1$  through targeted disruption of the gene *MpFlv1* (Fig. 44). We found that the maximum quantum efficiency of PSII photochemistry ( $F_v/F_m$ ) in  $\Delta MpFlv1$  was not different from that of the wild type Tak-1 or of the complement mutant to  $\Delta MpFlv1$  ( $cMpFlv1$ ) (Table 8). Additionally, total amounts of Chl *a* and *b* were almost the same among these plants (Table 8). Furthermore, the total nitrogen content of  $\Delta MpFlv1$  was also at the same level as those in Tak-1 and  $cMpFlv1$  (Table 8). These data indicate that the deletion of *MpFlv1* has little effect on the growth of *M. polymorpha* under the growth conditions used in this study as shown in a photo of these plants (Fig. 45).

To investigate the physiological functions of FLV in the protection of PSI and PSII in *M. polymorpha*, we illuminated the thalli of Tak-1,  $\Delta MpFlv1$ , and  $cMpFlv1$  with short-saturation pulse (SP). Repetitive SP illumination (rSP treatment) saturates the photosynthetic electron transport system with electrons to selectively inactivate PSI, with  $O_2$  required in  $C_3$  plant leaves (Sejima et al., 2014; Zivcak et al., 2015; Zivcak et al., 2015). In sunflower leaves,

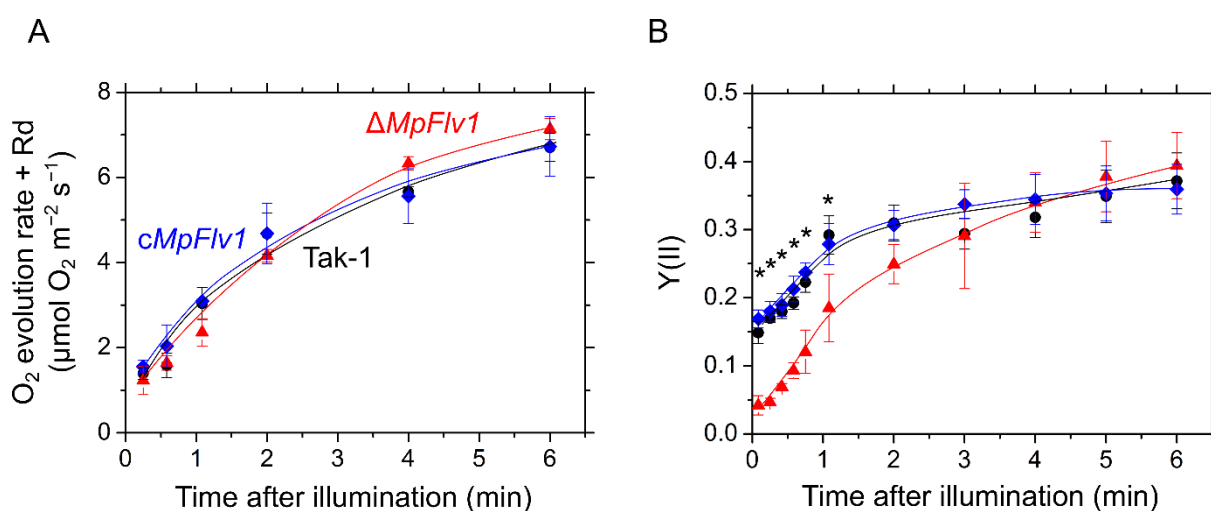
$P_m$  decreased to about 10% of the initial value through rSP treatment (300 ms, 20,000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , every 10 s) for 4 h, whereas  $F_v/F_m$  decreased to about 80% (Sejima et al., 2014). Hence, rSP treatment is a useful method of inducing photoinhibition of PSI *in vivo* and of investigating the robustness of PSI against photo-oxidative damage.



**Fig. 46.** Effects of rSP treatment on  $P_m$  (A) and  $F_v/F_m$  (B) in Tak-1,  $\Delta\text{MpFlv1}$ , and  $c\text{MpFlv1}$  under ambient air (respective dark-colored bars) and pure  $\text{N}_2$  gas (respective light-colored bars). These values were obtained 30 min (in the dark) after 15 min-treatments. For rSP treatments, repetitive short-saturation pulses ( $3,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 1 s) were applied every 10 s in the dark. Data are represented as the mean  $\pm$  SD of six independent measurements. Differences between Tak-1 and  $\Delta\text{MpFlv1}$  were analyzed using the Student's *t*-test. Asterisks indicate statistically significant differences between Tak-1 and  $\Delta\text{MpFlv1}$  at  $p < 0.05$ .

In  $\Delta\text{MpFlv1}$ , rSP treatment (1 s,  $3,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , every 10 s) significantly decreased both  $P_m$  and  $F_v/F_m$ , as compared to its effect in Tak-1 (Fig. 46A and B), which suggests that photoinhibition of PSI and PSII occurs during rSP treatment in  $\Delta\text{MpFlv1}$ . Post-treatment  $P_m$  and  $F_v/F_m$  were measured after a 30 min incubation in the dark to relax the

influence of NPQ, state transition, and chloroplast movements. Complementation of  $\Delta MpFlv1$  with  $MpFlv1$  partially relieved photoinhibition of both PSI and PSII (Fig. 46A and B). In  $\Delta MpFlv1$ , the degree of decrease in  $P_m$  was greater than that in  $F_v/F_m$  (Fig. 46A and B), indicating that, in liverworts as in  $C_3$  plants, rSP treatment leads to photo-oxidative damage mainly in PSI (Sejima et al., 2014). Compared with rSP treatment in air, the extent of PSI damage was smaller in  $\Delta MpFlv1$  during treatment in the absence of  $O_2$  (Fig. 46A and B), which suggests that photoinhibition of PSI in  $\Delta MpFlv1$  is caused by ROS. In contrast, the decrease in  $F_v/F_m$  during rSP treatment was accelerated in the absence of  $O_2$  (Fig. 46B). In PSII,  $O_2$ -insensitive photodamage might occur during rSP treatment (Krause et al., 1985).

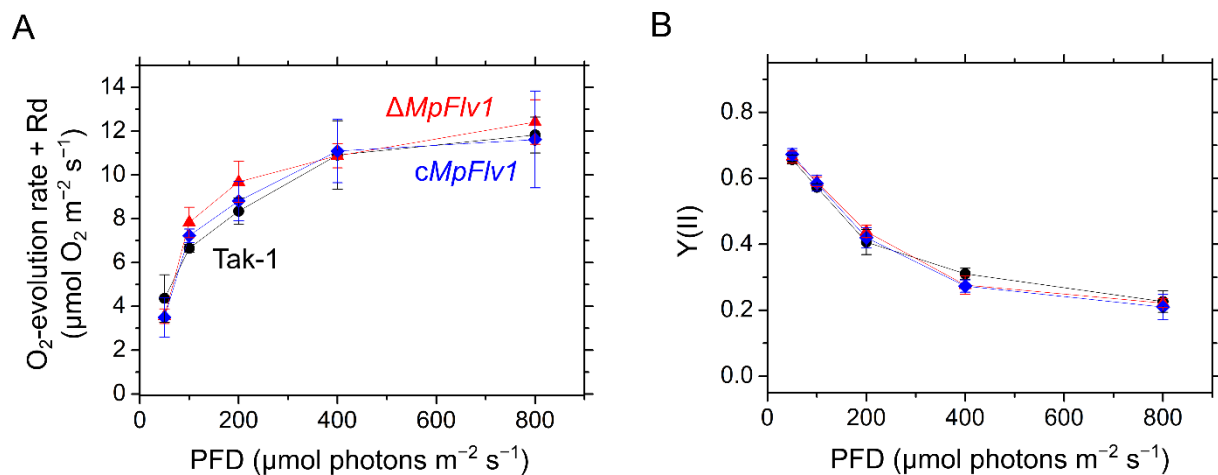


**Fig. 47.** Time course of total photosynthetic  $O_2$ -evolution rate (A) and  $Y(II)$  (B) in the induction phase of photosynthesis in Tak-1 (black circles),  $\Delta MpFlv1$  (red triangles), and  $cMpFlv1$  (blue diamonds).  $CO_2$ -saturated conditions were generated by adding 1 M  $NaHCO_3$  to the felt mat in the reaction chamber. Actinic light ( $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) was turned on at the zero time point. Data are represented as the mean  $\pm$  SD of three independent measurements. Differences in  $Y(II)$  between Tak-1 and  $\Delta MpFlv1$  were analyzed using the Student's  $t$ -test. Asterisks indicate statistically significant differences between Tak-1 and  $\Delta MpFlv1$  at  $p < 0.05$ .

## Effects of FLV on photosynthesis in *M. polymorpha*

As shown in Fig. 47A, FLV is required to protect PSI against photo-oxidative damage, which implies that the functions of FLV-mediated AEF in *M. polymorpha* are similar to those in cyanobacteria. To test this hypothesis, we simultaneously monitored O<sub>2</sub> exchange and Y(II) in Tak-1,  $\Delta MpFlvI$ , and  $cMpFlvI$  (Fig. 48). Before illumination with AL, we determined Rd to be  $1.5 \pm 0.5$ ,  $1.7 \pm 0.7$ , and  $1.6 \pm 0.5$   $\mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$  in the thalli of Tak-1,  $\Delta MpFlvI$ , and  $cMpFlvI$ , respectively ( $n = 6$ ). Upon illumination of the thalli, total photosynthetic O<sub>2</sub>-evolution rates [net O<sub>2</sub>-evolution rates (A) + Rd] gradually increased during photosynthetic induction. Similarly to Tak-1,  $\Delta MpFlvI$  showed an induction of photosynthesis (Fig. 48A). Furthermore,  $cMpFlvI$  also showed the same rate of photosynthetic induction as Tak-1 and  $\Delta MpFlvI$ . That is, the deficiency of *MpFlvI* did not affect photosynthesis. However, the behavior of Y(II) in these plants differed in the O<sub>2</sub>-evolution rate during the induction of photosynthesis. In Tak-1, Y(II) started to increase before the increase in the O<sub>2</sub>-evolution rate, just after the commencement of illumination (Fig. 47B). In contrast, in  $\Delta MpFlvI$ , the increase in Y(II) was delayed, and started to increase at 1 min after illumination was started. Y(II) in  $cMpFlvI$ , however, showed the same behavior as in Tak-1. These data indicate that, in *M. polymorpha*, the gene product of *MpFlvI* functions in AEF before the start of steady-state photosynthesis, which is also observed in the cyanobacterium S. 6803 (Fig. 48). Both gross photosynthetic O<sub>2</sub>-evolution rate and Y(II) in  $\Delta MpFlvI$  reached the same values as those in Tak-1 and  $cMpFlvI$  at steady-state photosynthesis (Fig. 47). We evaluated the dependences of the gross photosynthetic O<sub>2</sub>-evolution rate and Y(II) on photon flux density in Tak-1,  $\Delta MpFlvI$ , and  $cMpFlvI$  (Fig. 48). These data suggest that the effect of FLV on photosynthesis is smaller at the steady state, compared with the induction

phase.

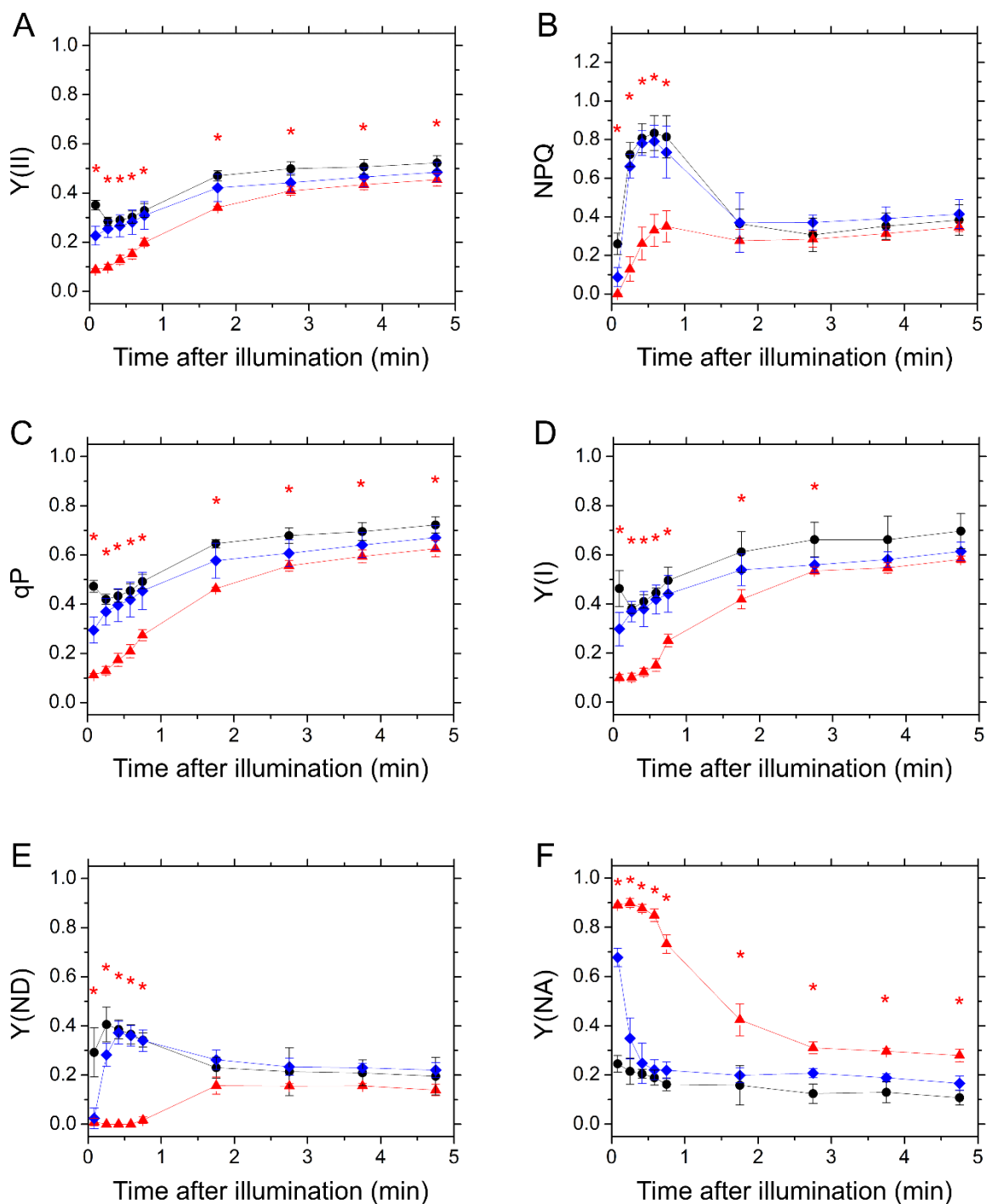


**Fig. 48.** Dependence of gross photosynthetic O<sub>2</sub>-evolution rates (A) and Y(II) (B) on photon flux density at steady-state photosynthesis in *Tak-1* (black circles),  $\Delta MpFlv1$  (red triangles), and *cMpFlv1* (blue diamonds). CO<sub>2</sub>-saturated conditions were generated by adding 1 M NaHCO<sub>3</sub> to the felt mat in the reaction chamber. Data are represented as the mean  $\pm$  SD of three independent measurements.

### Effects of FLV on photosynthetic parameters in PSII and PSI in *M. polymorpha*

In *M. polymorpha*, the gene product of *MpFlv1* mediates AEF (Fig. 47), and its absence accelerates photo-oxidative damage to PSI (Fig. 46A). In plant leaves and cyanobacterial cells, photoinhibition of PSI is alleviated by oxidation of P700, owing to a decrease in the amount of photo-oxidizable P700 (Sejima et al., 2014; Shimakawa et al., 2016). These data indicate that FLV-mediated AEF contributes to oxidation of P700 in *M. polymorpha*. To clarify the effects of FLV on the photosynthetic electron transport system in *M. polymorpha*, we investigated

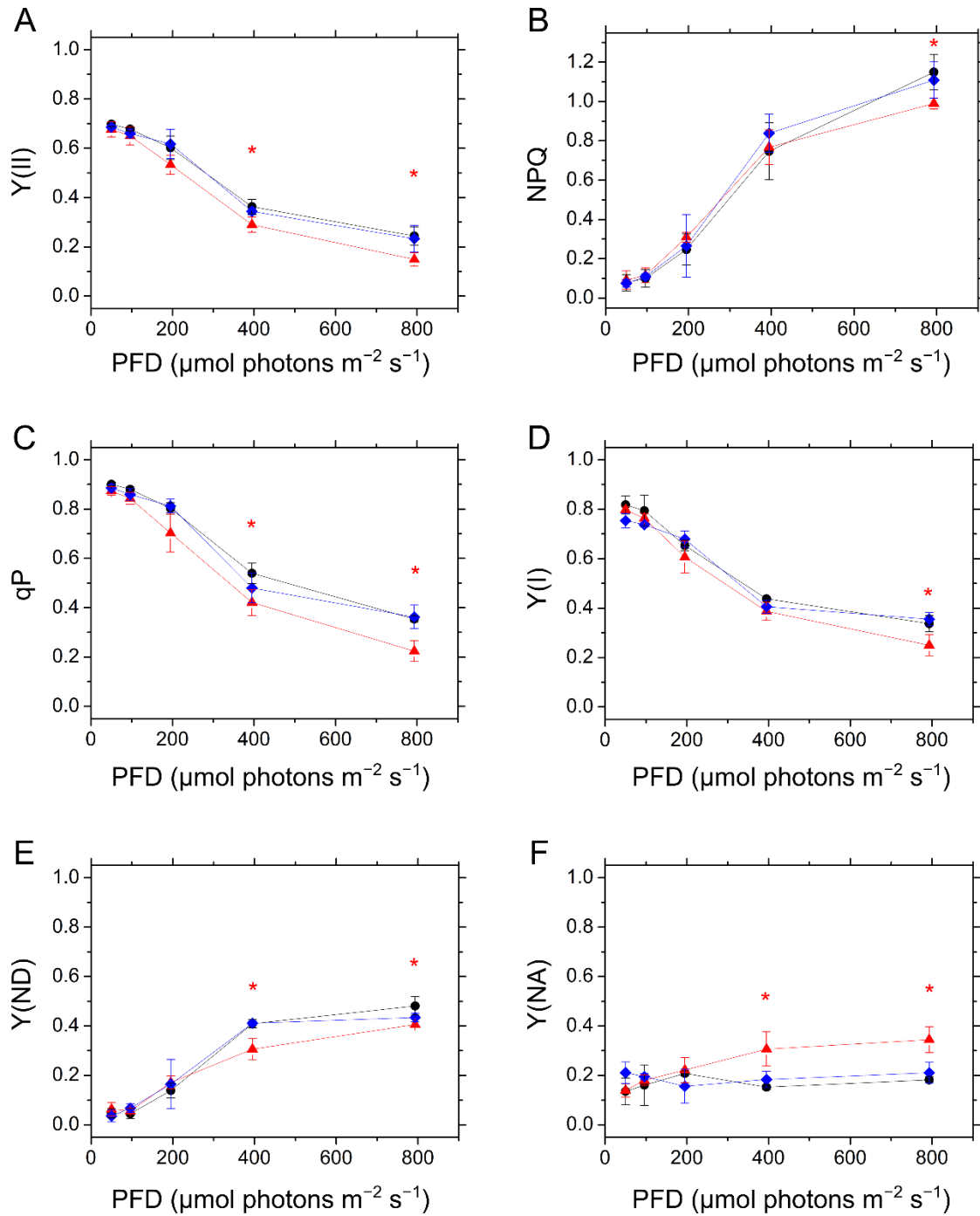
photosynthetic parameters in PSII and PSI in Tak-1,  $\Delta MpFlv1$ , and  $cMpFlv1$  in the response to illumination with AL.



**Fig. 49.** Time course of  $Y(II)$  (A), NPQ (B),  $qP$  (C),  $Y(I)$  (D),  $Y(ND)$  (E), and  $Y(NA)$  (F) in the induction phase of photosynthesis in Tak-1 (black circles),  $\Delta MpFlv1$  (red triangles), and

*cMpFlv1* (blue diamonds). Actinic light ( $195 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) was turned on at the zero time point. Measurements were taken under ambient air. Data are represented as the mean  $\pm$  SD of three independent measurements. Differences between *Tak-1* and  $\Delta\text{MpFlv1}$  were analyzed using the Student's *t*-test. Red asterisks indicate statistically significant differences between *Tak-1* and  $\Delta\text{MpFlv1}$  at  $p < 0.05$ .

First, we examined the photosynthetic parameters in PSII using Chl fluorescence measurement. In both *Tak-1* and *cMpFlv1*, Y(II) was higher than that in  $\Delta\text{MpFlv1}$  during the induction phase of photosynthesis (Fig. 49A). Thereafter, the Y(II) in  $\Delta\text{MpFlv1}$  became more similar to those in *Tak-1* and *cMpFlv1* with photosynthesis induction (Fig. 49A). Additionally, compared with  $\Delta\text{MpFlv1}$ , the observed NPQ was also higher in *Tak-1* and *cMpFlv1* in response to illumination with AL (Fig. 49B). Furthermore, the coefficient of photochemical quenching of Chl fluorescence (qP) was higher in *Tak-1* and *cMpFlv1* than in  $\Delta\text{MpFlv1}$  (Fig. 49C). The light-response curves of Y(II), NPQ, and qP during steady-state photosynthesis are also shown (Fig. 50A, B, and C). Similar to Y(II), NPQ and qP had almost the same values at steady-state photosynthesis in these three plants, with the exception of illumination with high light leading to lower Y(II), NPQ, and qP in  $\Delta\text{MpFlv1}$  than in *Tak-1* and *cMpFlv1* (Fig. 50A, B, and C). Some of the discrepancy between Y(II) values depicted in Fig. 48B and 50A might be due to differences in the equipment used for measuring Chl fluorescence. We can thus draw the following tentative conclusions from the foregoing results. FLV drives photosynthetic linear electron flow as AEF, which would induce  $\Delta\text{pH}$  to trigger NPQ. Both the stimulated electron sink and the enhanced NPQ caused by FLV-mediated AEF contribute to oxidation of the photosynthetic electron transport system, as demonstrated by the increase in qP (Fig. 49C and 50C) (Takizawa et al., 2007; Miyake, 2008).

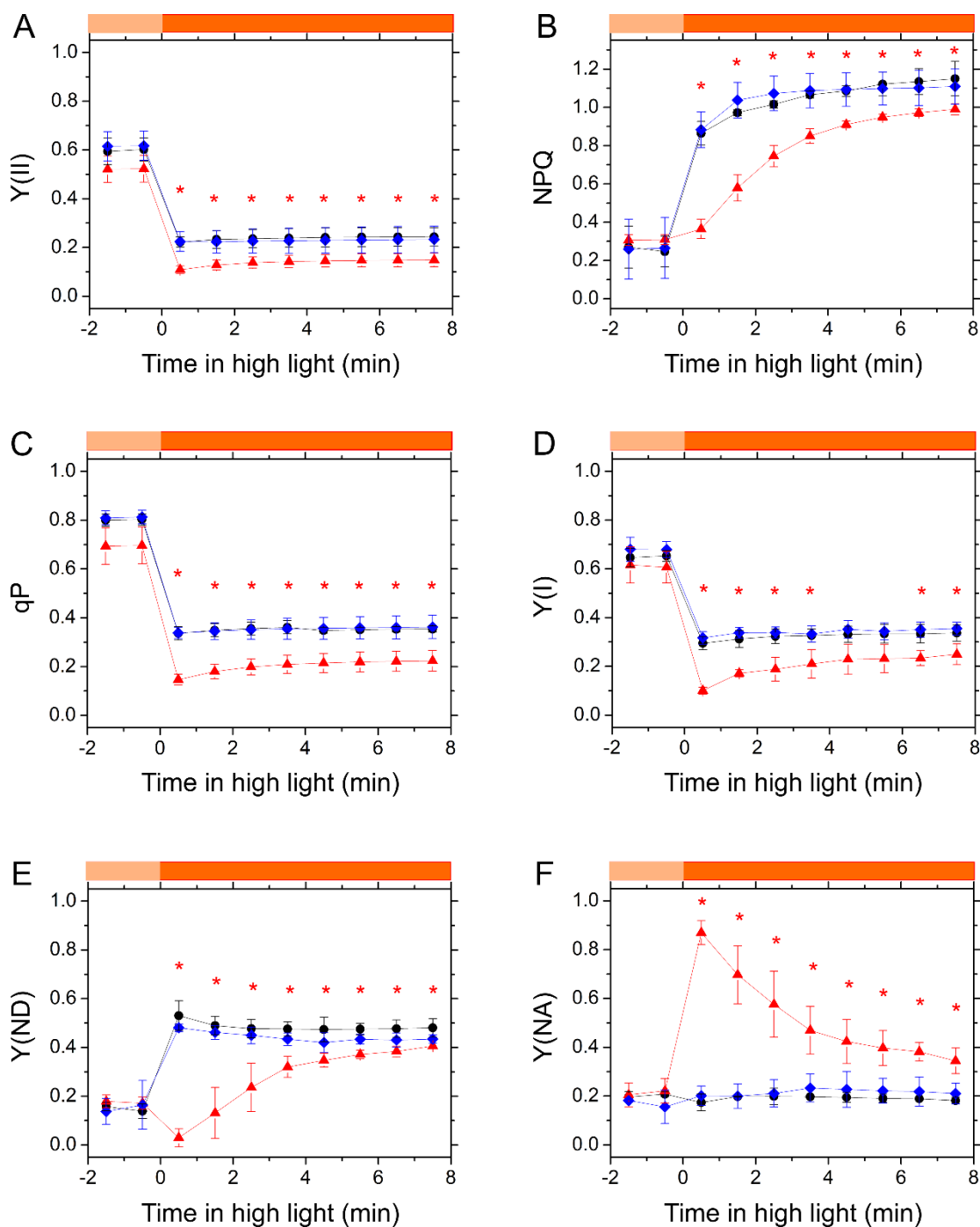


**Fig. 50.** Dependence of  $Y(II)$  (A),  $NPQ$  (B),  $qP$  (C),  $Y(I)$  (D),  $Y(ND)$  (E), and  $Y(NA)$  (F) on photon flux density at steady-state photosynthesis in *Tak-1* (black circles),  $\Delta\text{MpFlv1}$  (red triangles), and  $\text{cMpFlv1}$  (blue diamonds). Measurements were taken under ambient air. Data are represented as the mean  $\pm$  SD of three independent measurements. Differences between *Tak-*

*l* and  $\Delta MpFlv1$  were analyzed using the Student's *t*-test. Red asterisks indicate statistically significant differences between *Tak-1* and  $\Delta MpFlv1$  at  $p < 0.05$ .

Next, we investigated the effects of FLV on the photosynthetic parameters in PSI, evaluated by examining changes in absorbance of P700 (Klughammer and Schreiber, 1994, 2008; Schreiber and Klughammer, 2008). Similar to Y(II), we found higher quantum yields of PSI, Y(I), in *Tak-1* and *cMpFlv1* than in  $\Delta MpFlv1$  (Fig. 49D). We note that we cannot exclude the possibility that the deletion of FLV affects the distribution of PSII and PSI. Additionally, we measured the redox state of P700 in the induction phase of photosynthesis. Both *Tak-1* and *cMpFlv1* showed larger Y(ND) than  $\Delta MpFlv1$  (Fig. 49E), indicating that FLV-mediated AEF causes the donor-side limitation of PSI. In contrast, the acceptor-side limitations of PSI, Y(NA), were lower in *Tak-1* and *cMpFlv1* than in  $\Delta MpFlv1$  (Fig. 49F). That is, deletion of *MpFlv1* changed the limiting step from the donor side to the acceptor side of PSI. Thus, FLV contributes to the oxidation of P700 in the induction phase of photosynthesis in *M. polymorpha*, which might be responsible for the protection of PSI against photoinhibition (Fig. 46A). In *cMpFlv1*, lower Y(ND) and higher Y(NA) than those in *Tak-1* were observed just after starting illumination (Fig. 49E and F). The reason for this difference is unclear, but the recombinant gene product of *MpFlv1* in *cMpFlv1* might not function as well as the gene product expressed in *Tak-1*. The amount of the recombinant protein present in *cMpFlv1* also should be considered. The photosynthetic parameters, including Y(I), Y(ND), and Y(NA), in  $\Delta MpFlv1$  changed in a time-dependent manner to reach almost the same values as those in *Tak-1* and *cMpFlv1* at steady-state photosynthesis (Fig. 49D, E, and F). The light-response curves of Y(I), Y(ND), and Y(NA) during steady-state photosynthesis suggest that the contribution of FLV-mediated AEF to regulating the redox state of PSI is smaller at steady-state photosynthesis, compared with the

induction phase, although significant differences were found between Tak-1 and  $\Delta MpFlv1$  under high light (Fig. 58D, E, and F).



**Fig. 51.** Time course of  $Y(II)$  (A), NPQ (B),  $qP$  (C),  $Y(I)$  (D),  $Y(ND)$  (E), and  $Y(NA)$  (F) in the transition from moderate (200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , pale orange bars) to high (840  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , orange bars) light.

photons  $\text{m}^{-2} \text{s}^{-1}$ , orange bars) light in *Tak-1* (black circles), *ΔMpFlv1* (red triangles), and *cMpFlv1* (blue diamonds). Measurements were taken under ambient air. Data are represented as the mean  $\pm$  SD of three independent measurements. Differences between *Tak-1* and *ΔMpFlv1* were analyzed using the Student's *t*-test. Asterisks indicate statistically significant differences between *Tak-1* and *ΔMpFlv1* at  $p < 0.05$ .

Further, we measured photosynthetic parameters in PSII and PSI under a fluctuating light condition in *Tak-1*, *ΔMpFlv1*, and *cMpFlv1*. The thalli of these plants were illuminated with AL ( $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) to reach steady-state photosynthesis, and then we increased the photon flux density of AL by approximately 4 fold ( $840 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). During the transition from moderate to high light, Chl fluorescence and P700 absorbance were simultaneously monitored.

Both Y(II) and qP decreased in response to the transition to high light in all strains, with the largest decrease being observed in *ΔMpFlv1* (Fig. 51A and C). Thereafter, lowered Y(II) and qP in *ΔMpFlv1* gradually increased to reach constant values approximately 4 min after the change in photon flux density, although both Y(II) and qP were initially maintained at constant levels in *Tak-1* and *cMpFlv1* after the transition (Fig. 51A and C). Additionally, the induction of NPQ by illumination with high light was significantly retarded in *ΔMpFlv1* compared to its induction in the other strains (Fig. 51B). The behaviors of these photosynthetic parameters in PSII were consistent with those in photosynthesis induction (Fig. 49A, B, and C).

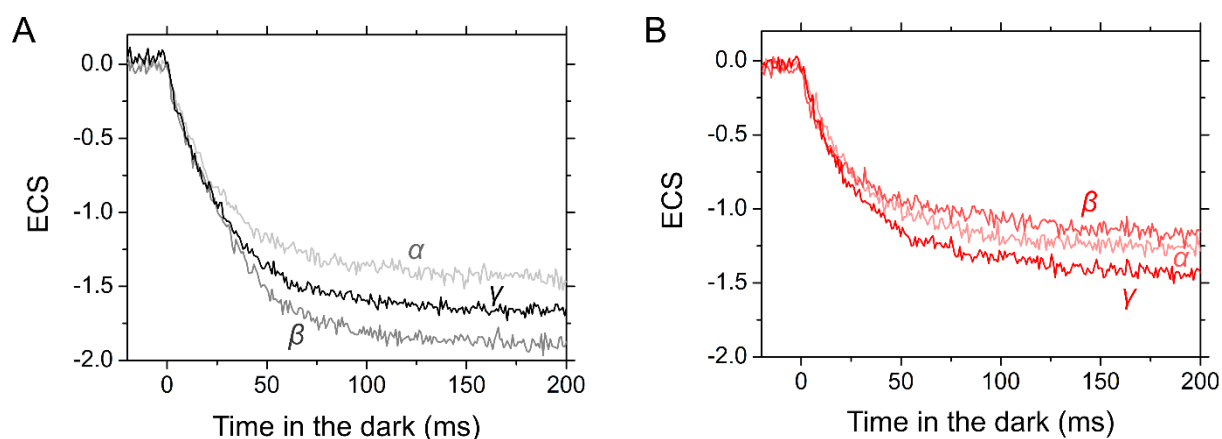
In the case of PSI, the decrease in Y(I) during the transition from moderate to high light was largest in *ΔMpFlv1* (Fig. 51D), similar to the responses of Y(II) and qP (Fig. 51A and C). In both *Tak-1* and *cMpFlv1*, Y(ND) rapidly rose in response to high light, and then gradually decreased to a constant level (Fig. 51E). However, *ΔMpFlv1* showed a slower induction of

Y(ND) than was observed in Tak-1 and *cMpFlv1* (Fig. 51E). In contrast, a rapid increase in Y(NA) was observed in  $\Delta MpFlv1$  during the transition to high light, whereas in Tak-1 and *cMpFlv1*, Y(NA) was not affected by the change in photon flux density (Fig. 51F). These data suggest that FLV-mediated AEF is required to maintain P700 in an oxidized state under fluctuating light in *M. polymorpha*, which is similar to observations of the cyanobacterium *S. 6803* (Allahverdiyeva et al., 2013; Shimakawa et al., 2016).

### **Effects of FLV on thylakoid membrane potential in *M. polymorpha***

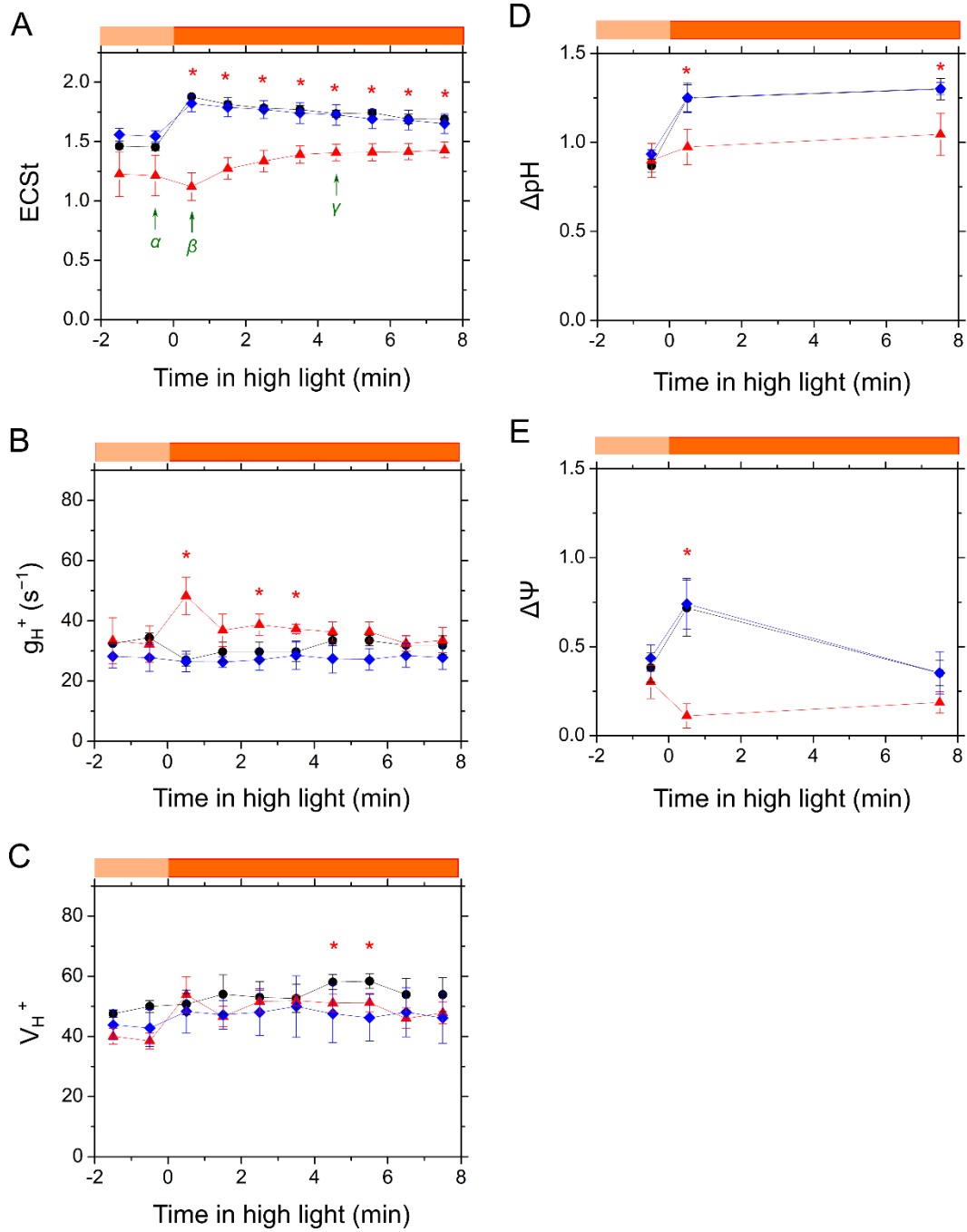
To clarify the relationship between the FLV-mediated AEF and P700 oxidation, we evaluated the effects of FLV on thylakoid membrane potential in *M. polymorpha* by analyzing electrochromic shift signals (ECS or P515, because the absorption measurements are made at 515 nm) (Klughammer et al., 2013) in the response to high light in Tak-1,  $\Delta MpFlv1$ , and *cMpFlv1*. The ECS signal is considered an intrinsic optical voltmeter that rapidly responds to changes in the electrical potential across the thylakoid membrane (Witt, 1971, 1979), and can be utilized in a non-invasive spectroscopic measurement (Cruz et al., 2005; Baker et al., 2007; Bailleul et al., 2010; Klughammer et al., 2013; Johnson and Ruban, 2014). The thylakoid membrane potential during photosynthesis is defined as the total rapid (<1 s) change in the ECS signal (ECSt) upon rapidly switching off AL from steady state, which reflects the light-dark difference in proton motive force, and includes two components: transmembrane differences in the concentration of protons (i.e.,  $\Delta pH$ ) and in the electric field ( $\Delta \Psi$ ) (Sacksteder et al., 2000; Cruz et al., 2001; Sacksteder et al., 2001; Cruz et al., 2005). In this study, the values of ECSt were normalized by dividing the magnitude of ECS decay in DIRK analysis by the magnitude of ECS induced by a 10  $\mu s$ -single turnover flash (Klughammer et al., 2013). The initial decay rate of the ECS signal following light to dark transitions can be used to estimate relative light-

driven proton flux through the chloroplast ATP synthase ( $H^+$ -efflux rate,  $V_H^+$ ), which is known to have a linear relationship with the photosynthetic linear electron transport rate only if there is no contribution to proton flux from CEF around PSI (Avenson et al., 2004; Avenson et al., 2005). Furthermore, the first-order decay time of the ECS decay in the light to dark transitions is required for the estimation of proton conductance in the ATP synthase ( $g_H^+$ ) (Kanazawa and Kramer, 2002; Rott et al., 2011; Kohzuma et al., 2013). We note that the ECS parameters are dependent on the properties of the leaves, not only the density of chloroplasts, but also the content of light-harvesting complexes that house the shifted pigments.



**Fig. 52.** The representative original traces of dark-interval relaxation kinetics of ECS in *Tak-1* (A) and  $\Delta MpFlv1$  (B) during the experiment shown in Fig. 53A–C. The shown traces were obtained during the transition to high light at  $-0.5$ ,  $0.5$ , and  $4.5$  min on the x-axis in Fig. 53A–C ( $\alpha$ ,  $\beta$ , and  $\gamma$  respectively). Line colors become darker over time. AL was turned off at time zero.

A lack of FLV had some impact on the ECS parameters during the transition to high light in *M. polymorpha*. The representative original kinetics of the ECS signal following light to dark transitions in *Tak-1* and  $\Delta MpFlv1$  are shown in Fig. 52, and were utilized for estimating



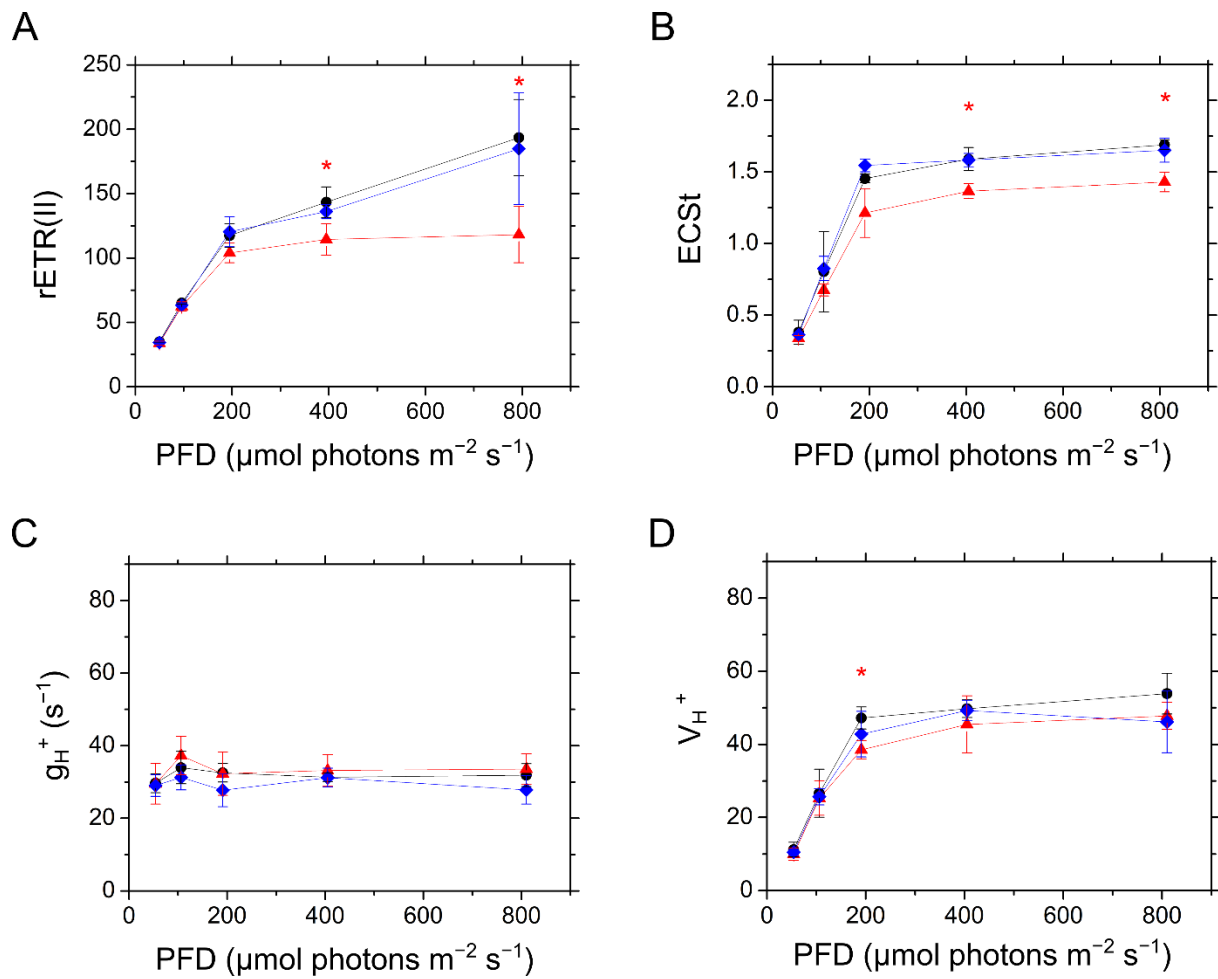
**Fig. 53.** Time courses of ECSt (A),  $g_H^+$  (B),  $V_H^+$  (C),  $\Delta pH$  (D), and  $\Delta \Psi$  (E) in the transition from moderate ( $180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , pale orange bars) to high ( $760 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , orange bars) light in Tak-1 (black circles),  $\Delta$ MpFlv1 (red triangles), and cMpFlv1 (blue diamonds). Measurements were taken under ambient air. Data are represented as the mean  $\pm$  SD of three independent measurements. Differences between Tak-1 and  $\Delta$ MpFlv1 were analyzed

using the Student's *t*-test. Red asterisks indicate statistically significant differences between *Tak-1* and  $\Delta MpFlv1$  at  $p < 0.05$ . The original traces of the ECS signal at  $\alpha$ ,  $\beta$ , and  $\gamma$  (indicated by green arrows) are shown in Fig. 52.

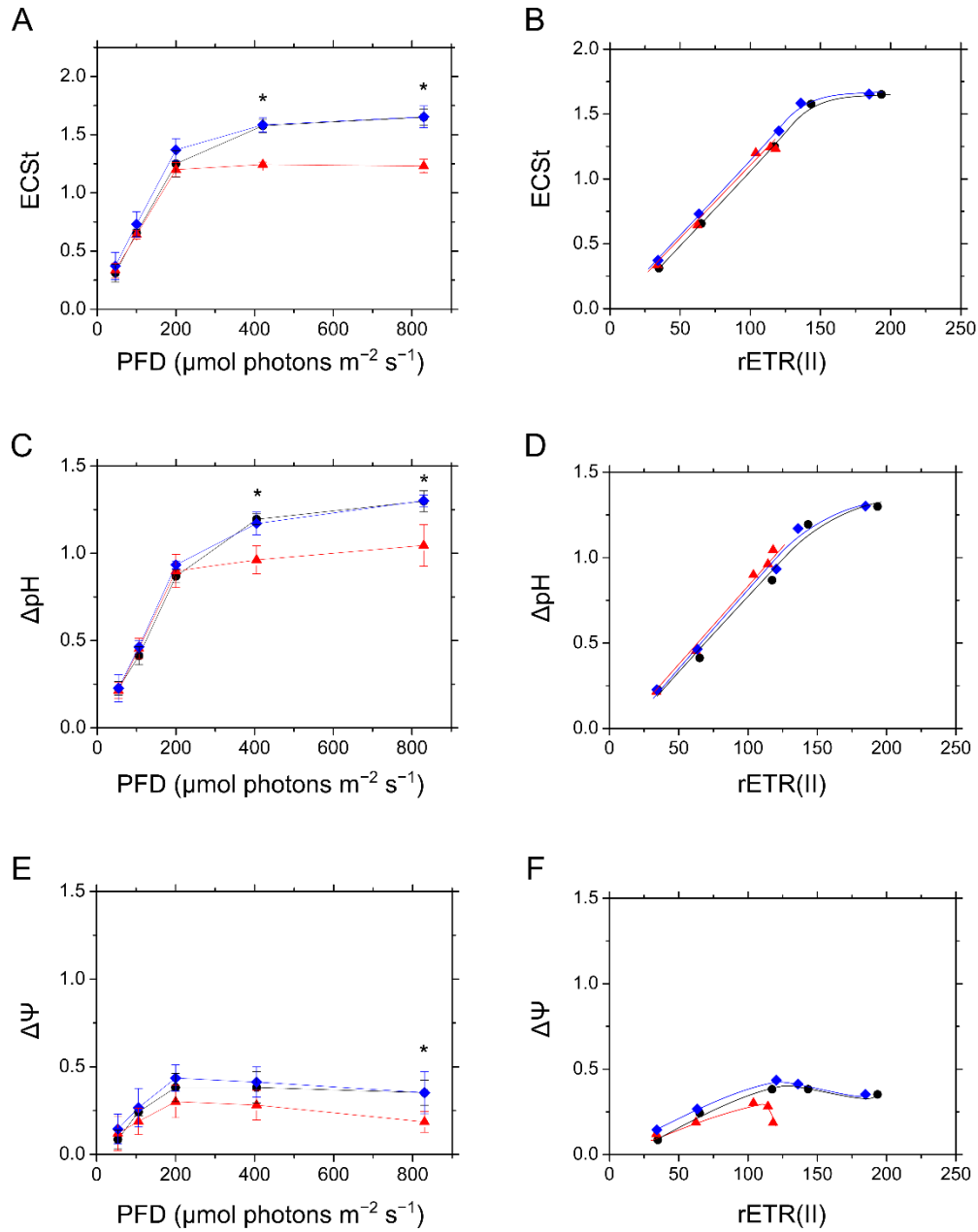
the ECS parameters of the following results. In response to high light, ECSt in *Tak-1* and *cMpFlv1* first rapidly increased, and thereafter gradually decreased (Fig. 53A). Compared with *Tak-1* and *cMpFlv1*, ECSt in  $\Delta MpFlv1$  decreased just after the start of illumination with high light, which was accompanied by an increase in  $g_H^+$ , thereafter gradually increased (Fig. 53A and B). We divided ECSt into  $\Delta pH$  and  $\Delta \Psi$  following the method described by Klughammer et al. (2013), indicating that in *M. polymorpha* FLV-mediated AEF stimulates LEF to support the establishment of  $\Delta pH$  (Fig. 53D and E). It is still unclear why  $g_H^+$  increased in  $\Delta MpFlv1$  just after the thalli were exposed to high light (Fig. 53B). In contrast,  $V_H^+$  slightly increased in response to high light in all three of the plants (Fig. 53C).

We evaluated the dependences of the relative ETR at PSII, i.e., rETR(II), calculated as the product of Y(II) and photon flux density, and ECS parameters on photon flux density during steady-state photosynthesis in *Tak-1*,  $\Delta MpFlv1$ , and *cMpFlv1* (Fig. 54 and 55), which shows the increase in rETR(II) uncoupled with ECSt in the range of PFD approximately over 200  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Unfortunately, we could not determine the reasons for the gap between ECSt and rETR(II) in *M. polymorpha*. In intact chloroplasts and plant leaves, CEF within PSII drives in sensitive to  $\Delta pH$  to oxidize the PQ pool (Miyake and Yokota, 2001; Miyake et al., 2002; Laisk et al., 2006). In *M. polymorpha*, FLV-mediated AEF might function in the induction of CEF within PSII through the formation of  $\Delta pH$ , resulting in the increases in Y(II) and qP (Fig. 50C, 54A and B, and 55A–D). The other possibility is that changes in the ratio between components of the photosynthetic machinery, including state transition, might be related to

these results.

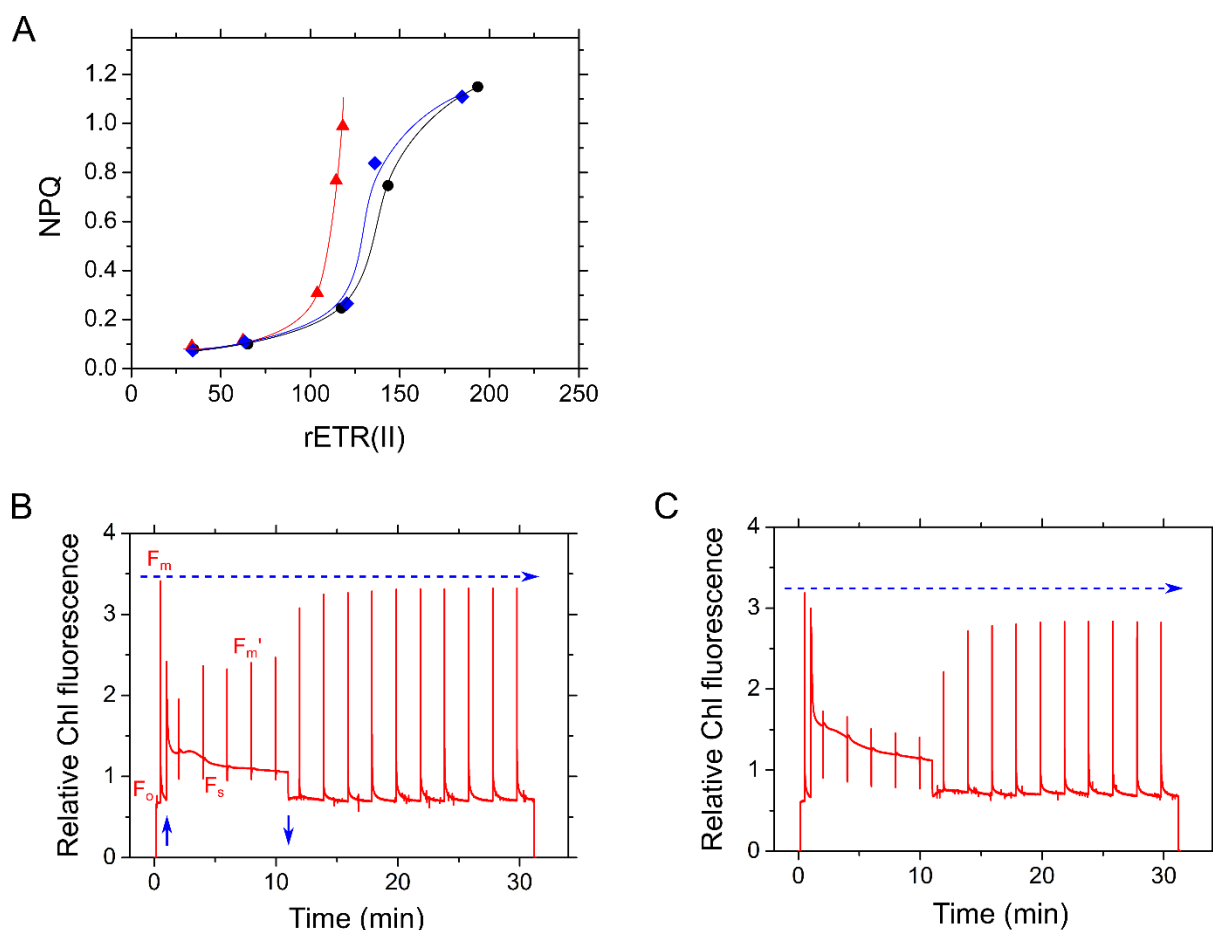


**Fig. 54.** Dependence of rETR (A), ECSt (B),  $g_H^+$  (C), and  $V_H^+$  (D) on PFD at steady-state photosynthesis in Tak-1 (black circles),  $\Delta\text{MpFlv1}$  (red triangles), and cMpFlv1 (blue diamonds). Measurements were taken in ambient air. Calculation of rETR(II) was conducted using the data from Fig. 50A. Data are represented as the mean  $\pm$  SD of three independent measurements. Differences between Tak-1 and  $\Delta\text{MpFlv1}$  were analyzed using Student's *t*-test. Red asterisks indicate statistically significant differences between Tak-1 and  $\Delta\text{MpFlv1}$  at  $p < 0.05$ .



**Fig. 55.** Dependence of ECSt (A),  $\Delta\text{pH}$  (C), and  $\Delta\Psi$  (E) on PFD and the relationship of ECSt (B),  $\Delta\text{pH}$  (D), and  $\Delta\Psi$  (F) to rETR(II) at steady-state photosynthesis in *Tak-1* (black circles),  $\Delta$ MpFlv1 (red triangles), and cMpFlv1 (blue diamonds). Measurements were taken in ambient air. Calculation of the rETR(II) was conducted using the data of Fig. 50A. Respective ECS parameters were plotted on the rETR(II) at approximately the same PFD. Data are represented as the mean  $\pm$  SD of three independent measurements. Differences between *Tak-1* and  $\Delta$ MpFlv1 were analyzed using Student's *t*-test. Asterisks indicate statistically significant differences

between *Tak-1* and  $\Delta$ MpFlv1 at  $p < 0.05$ .

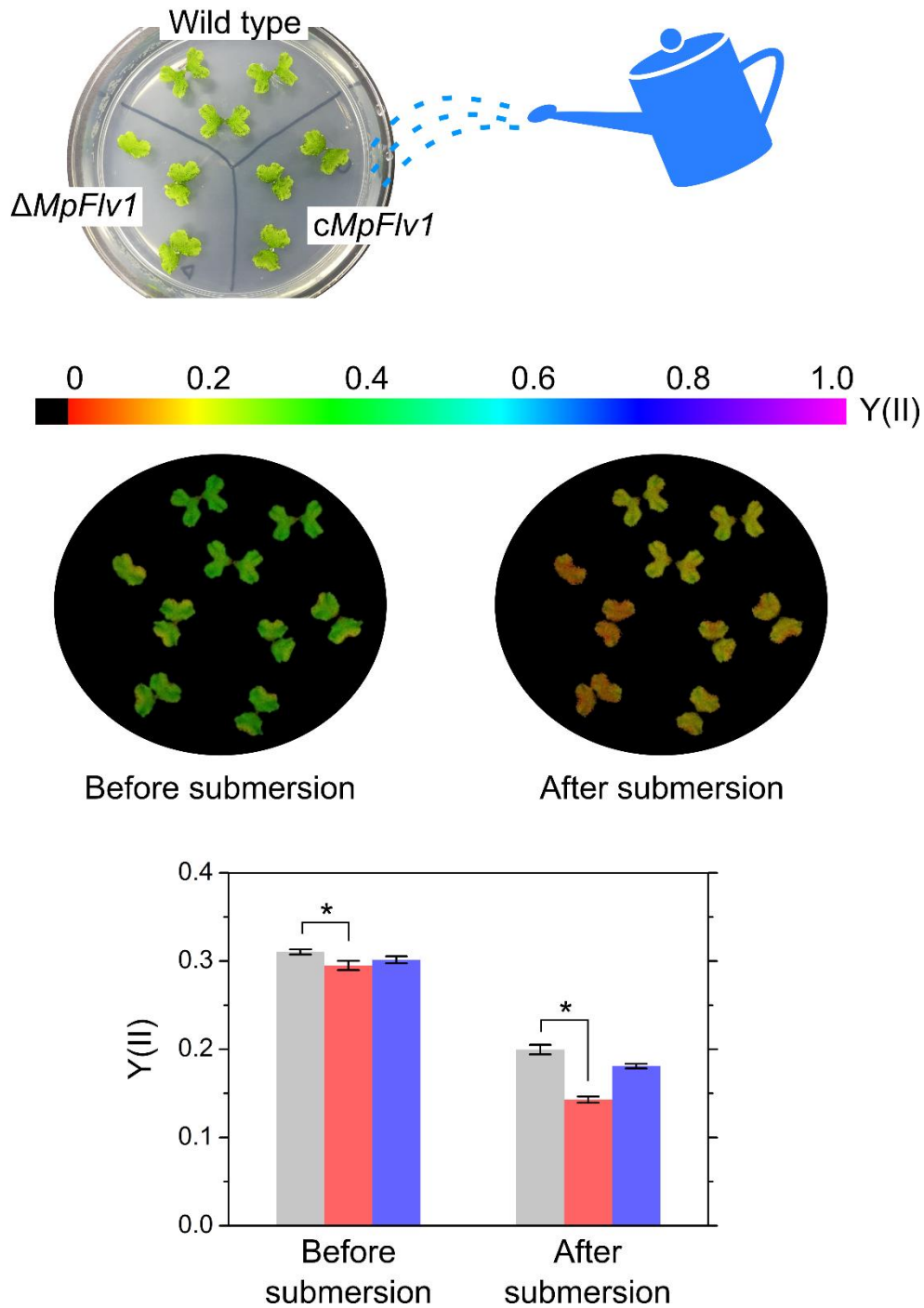


**Fig. 56.** (A) Relationship of NPQ to rETR(II) at steady-state photosynthesis in *Tak-1* (black circles),  $\Delta$ MpFlv1 (red triangles), and cMpFlv1 (blue diamonds). Measurements were taken in ambient air. Calculation of rETR(II) was conducted using the data from Fig. 50A. NPQ from Fig. 50B was plotted on rETR at the same PFD. Data are represented as the mean  $\pm$  SD of three independent measurements. (B, C) Relaxation of NPQ after transition to the dark in *Tak-1*. Blue dashed lines indicate the  $F_m$  levels. Up and down blue arrows show the times that AL was turned on and off, respectively. PFD of AL was adjusted to 190 (A) and 760 (B)  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Representative data of three independent measurements were shown.

We also evaluated the relationship of NPQ with rETR(II) during steady-state photosynthesis in Tak-1,  $\Delta MpFlv1$ , and  $cMpFlv1$ . In plant leaves, NPQ is observed mainly in the form of qE, which is proportional to  $\Delta pH$  but not to  $\Delta \Psi$ , and involves a fast (seconds to a few minutes time scale) PSII antenna reorganization (Avenson et al., 2004; Takizawa et al., 2007; Takizawa et al., 2008). Nevertheless, NPQ did not show the linear relationship with rETR(II) in all three strains of *M. polymorpha* (Fig. 56A) in the range of rETR that was proportional to  $\Delta pH$  (Fig. 55D). We measured the time scale of the relaxation of NPQ after turning off of AL in Tak-1 (Fig. 56B and C), which suggests that  $\Delta pH$ -insensitive NPQ, including qT and qI (Grieco et al., 2012; Bergner et al., 2015; Derks et al., 2015; Wientjes et al., 2017), might occur, particularly under high light, in *M. polymorpha*.

### **Response of PSII operating efficiency to a submerged condition in *M. polymorpha***

We measured the responses of Y(II) to a submerged condition in Tak-1,  $\Delta MpFlv1$ , and  $cMpFlv1$  using an imaging-PAM. *M. polymorpha* thalli on agar were illuminated with blue AL. After photosynthesis reached steady state, we added distilled water to completely submerge the thalli in water. During the transition to a submerged condition, decreases in Y(II) were observed in all three strains, though the change was most prominent in  $\Delta MpFlv1$  (Fig. 57). The diffusion efficiency of CO<sub>2</sub> is approximately 10<sup>4</sup> times lower in aqueous environments compared with in the atmosphere (Armstrong, 1980; Raven et al., 1985; Pedersen et al., 2009; Sasidharan et al., 2017). Submergence limits CO<sub>2</sub> supply to Rubisco in chloroplasts. This suppressed photosynthesis would stimulate the FLV-mediated AEF.



**Fig. 57.** Responses of  $Y(II)$  to a submersion in *Tak-1*,  $\Delta MpFlv1$ , and  $cMpFlv1$ . Representative light field- and fluorescence-images of thalli on an agar medium are shown. Fluorescence images showing  $Y(II)$  were taken before and 20 s after submersion at the steady-state photosynthesis. PFD of blue AL was adjusted to  $240 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ .  $Y(II)$  in *Tak-1* (light grey bars),  $\Delta MpFlv1$  (pale red bars), and  $cMpFlv1$  (pale blue bars) before and 20 s after

*submersion at the steady-state photosynthesis. Data are represented as the mean  $\pm$  SD of three independent measurements. Differences between Tak-1 and  $\Delta$ MpFlv1 were analyzed using the Student's *t*-test. Asterisks indicate statistically significant differences between Tak-1 and  $\Delta$ MpFlv1 at  $p < 0.05$ .*

#### 7.4. Concluding remarks

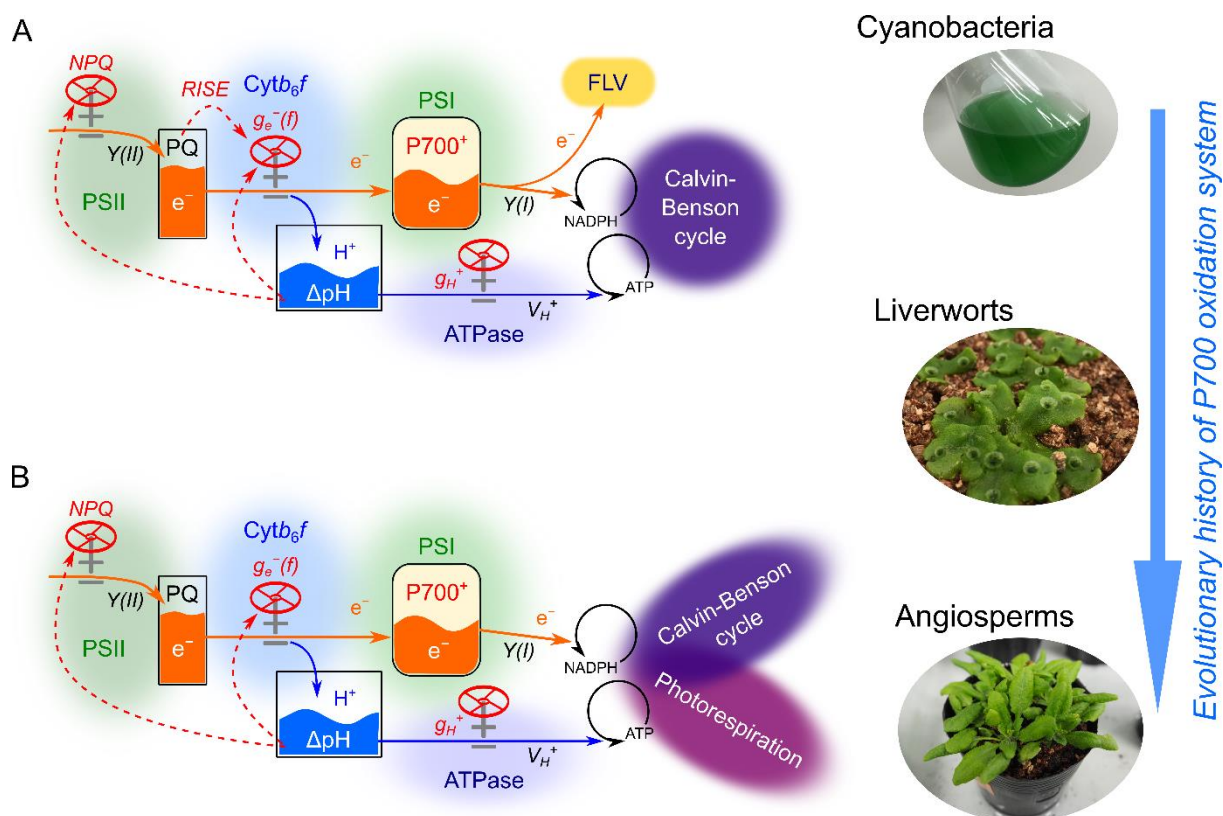
We clarified the physiological functions of FLV in the liverwort *M. polymorpha*, which has two genes homologous to *flv1* and 3 found in the cyanobacterium S. 6803. In S. 6803, most of the LEF can be passed to FLV1/3 in photosynthesis induction (Helman et al., 2003; Helman et al., 2005; Allahverdiyeva et al., 2011; Hayashi et al., 2014), and the FLV-mediated AEF partially proceeds at steady-state photosynthesis (Helman et al., 2005; Schuurmans et al., 2015; Schuurmans et al., 2017). Similar to FLV in S. 6803, the gene product of *MpFlv1* drives AEF in *M. polymorpha*. In the induction phase of photosynthesis, the electron flux mediated by FLV is estimated to contribute to approximately one-quarter of the linear electron flow, at least at the PFD we used in this study (Fig. 47), which is smaller than that observed in S. 6803. Nevertheless, FLV-mediated AEF contributes to the oxidation of the PQ pool (Fig. 49C and 59C) and P700 (Fig. 49E and 51E). An absence of *MpFlv1* promotes photoinhibition of PSI and PSII in *M. polymorpha* (Fig. 46). These results indicate that the physiological roles of FLV have been conserved at the current evolutionary stage of basal land plants.

Liverworts probably require FLVs and their functions due to the environmental conditions of their habitats, which is implied by the results in Fig. 57. Land plants are exposed to CO<sub>2</sub> limitations when submerged, because the diffusion rate of CO<sub>2</sub> in the water is much lower than in the atmosphere (Brestic et al., 1995). Generally, in C<sub>3</sub> plants exposed to low CO<sub>2</sub> conditions, photorespiration functions as an alternative electron sink to replace photosynthesis (Kozaki and Takeba, 1996; Badger et al., 2000; Takahashi et al., 2007; Sejima et al., 2016; Hanawa et al., 2017), which contributes to the dissipation of excess photon energy and suppresses photo-oxidative damages. However, in aqueous conditions, the efficiency of ribulose 1,5-bisphosphate (RuBP) oxygenation catalyzed by Rubisco is low, because the affinity of RuBP oxygenation reactions for O<sub>2</sub>,  $K_m$  for O<sub>2</sub>, ranges from 250 to 450  $\mu$ M O<sub>2</sub>

(Jordan and Ogren, 1981; Ogren, 1984). The concentration of O<sub>2</sub> in the water equilibrated with the atmosphere is about 250 μM at 25 °C. These facts imply the photorespiration rate is limited by the supply of O<sub>2</sub> from the atmosphere, and Rubisco cannot turn over at the maximum rate under these conditions. On the other hand,  $K_m$  for O<sub>2</sub> in FLV reactions is below a few μM O<sub>2</sub> (Vicente et al., 2002; Shimakawa et al., 2015) and FLV catalyzes the reduction of O<sub>2</sub> to water at its maximum rate in the water. In fact, in some species of cyanobacteria (S. 6803, S. 7942, and S. 7002), under suppressed photosynthesis conditions equilibrated with air, AEF is driven by FLVs, not by photorespiration (see the “Part I” and “Part III”) (Hayashi et al., 2014; Shimakawa et al., 2015; Shimakawa et al., 2016; Shimakawa et al., 2016). Furthermore, some eukaryotic algae, including the green alga *Chlamydomonas reinhardtii* and the diatom *Phaeodactylum tricornutum*, do not utilize photorespiration as the main alternative electron sink under suppressed photosynthesis conditions (see the “Part I” and “Part II”) (Roach et al., 2015; Shimakawa et al., 2016; Chaux et al., 2017; Shimakawa et al., 2017). These facts suggest that oxygenic phototrophs that reside in or are exposed to aqueous environments use FLV in AEF to oxidize P700 in PSI. Recently, the moss *Physcomitrella patens* was reported to show FLV-mediated AEF, similarly to *M. polymorpha* (Gerotto et al., 2016). Further, FLV1/3 is proposed to function in ferns and gymnosperms (Ilik et al., 2017; Noridomi et al., 2017; Takagi et al., 2017). Physiological functions of FLV may be conserved in whole basal land plants.

Here, we propose a model of the mechanisms involved in oxidizing P700 for suppression of photo-oxidative damages in PSI derived from ROS in the liverwort *M. polymorpha* (Fig. 58), which appears to be broadly applicable to various oxygenic phototrophs, with the exception of the extent of FLV activity. In angiosperms, which generally do not possess FLV, this mechanism might be replaced with other molecular mechanisms, including the photorespiratory C<sub>2</sub> cycle, PTOX, the Mehler-ascorbate peroxidase pathway (i.e., water-water cycle), CEF (including NDH and ferredoxin-quinone reductase), and malate dehydrogenase.

Additionally, the electron transport in Cyt  $b_6/f$  is also suppressed by sensing the redox state of the PQ pool in the RISE system, which has been characterized in the cyanobacterium S. 7942 (Shaku et al., 2016; 釋啓一郎 and 三宅親弘, 2016), but not yet in photosynthetic eukaryotes. In RISE system, accumulation of the reduced form of PQ inhibits the Q-cycle in Cyt  $b_6/f$ . That is, RISE can oxidize P700, where no limitation of acceptor side of PSI is required as prerequisite. The physiological functions of RISE in *M. polymorpha* await investigation in future studies. We note that there should be strategic diversity in oxygenic phototrophs commensurate with their survival on the earth.



**Fig. 58.** A summary diagram of P700 oxidation system in oxygenic phototrophs and its evolutionary history from cyanobacteria to angiosperms. The term  $g_e^-(f)$  indicates the conductance of electron transport in Cyt  $b_6/f$ .

## 8. Comprehensive Conclusion

In the doctoral research, I mainly contributed to establishing the novel concept “P700 oxidation system” in our laboratory. Oxidation of P700 has been broadly known as a physiological response of oxygenic phototrophs to excess light conditions (Badger and Schreiber, 1993; Klughammer and Schreiber, 1994; Golding and Johnson, 2003; Miyake et al., 2005), although the physiological significance is poorly understood. Additionally, the photo-oxidative damage in PSI (so-called PSI photoinhibition) was considered as an event only in specific situations such as isolated photosynthetic apparatus, cold stress, mutant plants (Satoh, 1970; Terashima et al., 1994; Munekage et al., 2002; Sonoike, 2011; Suorsa et al., 2012; Kono et al., 2014). The first step for establishing the novel concept is the report by Sejima et al. (2014) that showed the proportional relationship of P700 oxidation with the alleviation of the photo-oxidative damage in PSI against ROS in intact plant leaves, which has expanded the understanding of the protection mechanism of PSI from photoinhibition. Thereafter, we published the paper entitled “Oxidation of P700 in photosystem I is essential for the growth of cyanobacteria” in *Plant Physiology*, which includes several essential impacts on establishing the novel concept “P700 oxidation system” (Shimakawa et al., 2016) (see the “Part III”). First, we clearly showed that P700 oxidation is the universal lifeline for protecting PSI against photo-oxidative damage using different three cyanobacteria species. Second, we first evidenced that inability to oxidize P700 impairs the growth of oxygenic phototrophs. Third, the P700 oxidation strategy to alleviate PSI photoinhibition has already been developed at the time of the birth of cyanobacteria, which is known as the progenitor of photosynthetic organisms, and finally the strategy has already been diversified in the time. Recently, Takagi et al. (2017) has improved the method in Sejima et al. (2014) to clearly show the impacts of P700 oxidation on the protection of PSI against photoinhibition derived from ROS in plant leaves. From these reports, we have succeeded to

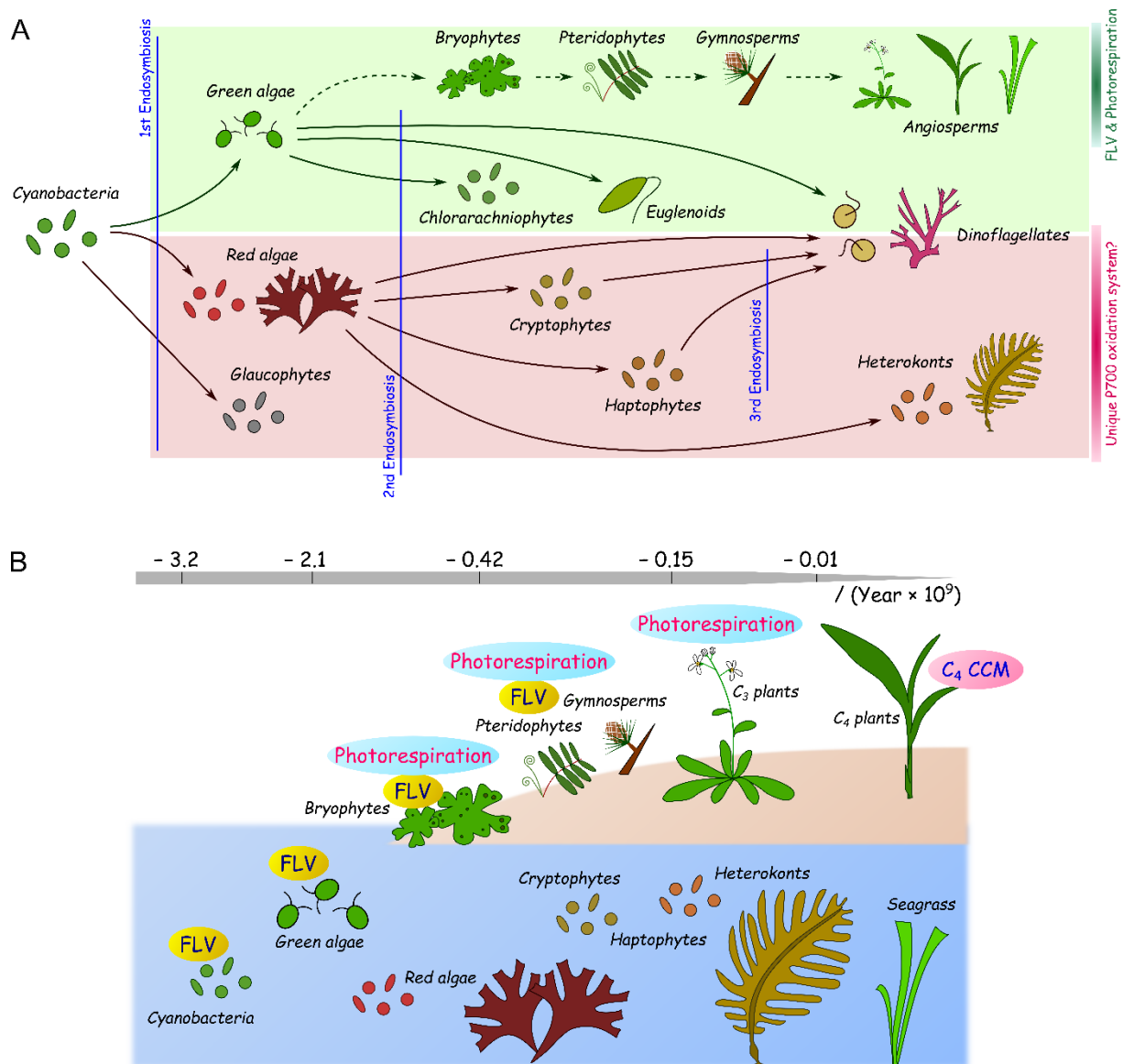
verify the physiological significance of P700 oxidation in oxygenic phototrophs. On the other hand, the detailed mechanism of the production of ROS in PSI still remain to be uncovered in spite of the recent progresses (Cazzaniga et al., 2012; Roach et al., 2015; Cazzaniga et al., 2016; Takagi et al., 2016). Qualitative and quantitative measurements of ROS may help the further understanding of the production mechanism of ROS in PSI.

Cyanobacteria, the pioneers of oxygenic photosynthesis, strongly depend on O<sub>2</sub>-use to alleviate the risk of O<sub>2</sub>-mediated damage. On the other hand, the electron transport in Cyt *b<sub>6</sub>/f* is suppressed sensitive to the reduction of PQ pool by RISE, an O<sub>2</sub>-insensitive P700 oxidation system found in S. 7942 (Shaku et al., 2016). In cyanobacteria, P700 oxidation requires the use of O<sub>2</sub> as an alternative electron acceptor in the FLV-mediated reactions on the acceptor sides of PSI. These agents for O<sub>2</sub>-use have the ability to utilize very small amounts of O<sub>2</sub> (a few or less μM O<sub>2</sub>) (Vicente et al., 2002; Hayashi et al., 2014; Shimakawa et al., 2015; Shimakawa et al., 2016), when compared with photorespiration of land plants (Jordan and Ogren, 1981; Ogren, 1984). In fact, both Cyd and FLV homologs in the anaerobic photosynthetic bacteria *Chlorobaculum tepidum* play a role in O<sub>2</sub> scavenging (Li et al., 2009). These facts suggest that cyanobacteria, which had first evolved O<sub>2</sub> on the earth, are profoundly afraid of the risk of O<sub>2</sub>, and strongly require the benefit of O<sub>2</sub>. Therefore, oxygenic photosynthesis would not likely have arisen without the benefits of O<sub>2</sub>-use.

I have focused mainly on O<sub>2</sub>-usage among diverse molecular mechanisms regulating P700 oxidation throughout the doctoral research (Fig. 59). We found that in C<sub>3</sub> plants, different from C<sub>4</sub> plants, photorespiration functions as the large alternative electron sink (Sejima et al., 2016), which was further expanded to a variety of land plants by Hanawa et al. (2017) showing that photorespiration is the dominant O<sub>2</sub>-dependent AEF in land plants (bryophytes, pteridophytes, gymnosperms, and angiosperms) except for C<sub>4</sub> plants harboring CO<sub>2</sub> concentrating mechanism (CCM). On the other hand, Takagi et al. (2016) reported that

photorespiration mainly contributes to P700 oxidation in the WT intact sunflower leaves by finely tuning O<sub>2</sub> partial pressure in the measuring system. Overall, photorespiration is the main P700 oxidation system on the electron acceptor side of PSI in land plants. Conversely, we have shown that the electron flux through photorespiration is very small, compared with photosynthesis and FLV reaction, in aquatic algae such as cyanobacteria, green algae, and diatoms possibly except for *E. gracilis* (see the “Part I and II”) (Hayashi et al., 2014; Shimakawa et al., 2015; Shaku et al., 2016; Shimakawa et al., 2016; Shimakawa et al., 2017). Instead, FLV functions as the dominant O<sub>2</sub>-usage for oxidizing P700 in cyanobacteria and algae (see the “Part III”) (Helman et al., 2003; Helman et al., 2005; Allahverdiyeva et al., 2013; Shimakawa et al., 2016). That is, the O<sub>2</sub>-use strategy has changed from FLV to photorespiration in the evolutionary history of oxygenic phototrophs from cyanobacteria to angiosperms (Fig. 58, see the “Part IV”) (Gerotto et al., 2016; Chaux et al., 2017; Shimakawa et al., 2017). Nevertheless, it should be noted that oxygenic phototrophs could advance to the terrestrial fields possibly owing to FLV (see the “Part IV”). On the other hand, it is still unclear how the other photosynthetic organisms than cyanobacteria, green algae, and land plants, oxidize P700 in excess light conditions. These oxygenic phototrophs include red algae, yellow-green algae, diatoms, and brown algae, and termed as photosynthetic “red plastid lineage” (Falkowski et al., 2004; Rademacher et al., 2016). The diatom *Phaeodactylum tricornutum* showed the O<sub>2</sub>-insensitive response of photosynthetic electron transport to CO<sub>2</sub> limitation (see the “Part II”), implying that red algae and red algae-derived secondary algae oxidize P700 without O<sub>2</sub>. Overall, the oxygenic phototrophs in the photosynthetic “red plastid lineage” might have developed a unique strategy to keep P700 oxidized in response to an excess light condition (Fig. 59). Although the activity should be strictly modulated in response to environmental variations, FLV is likely to have the potential to deprive the acceptor side of PSI of electrons for photosynthetic CO<sub>2</sub> assimilation. Additionally, FLV has a diiron site (Vicente et al., 2002), and is assumed to

spend much iron, which is slight in the ocean. Possibly, utilizing FLV is inconvenient for the acclimation to the habitats of these red plastid algae.



**Fig. 59.** Hypothetical illustration of the evolutionary history of the  $O_2$  use-strategy for P700 oxidation in oxygenic phototrophs.

Although various molecular mechanisms were reported as P700 oxidation system on

the acceptor side of PSI, the impacts of the donor-side mechanisms on P700 oxidation in cyanobacteria, algae, and plants remain to be investigated in detail. Recently, Shaku et al. (2016) reported the novel P700 oxidation system on the donor side of PSI (i.e., RISE) in *S. 7942*, but it is not characterized in photosynthetic eukaryotes yet. Basically, Cyt *b<sub>6</sub>/f* is known to be the limiting step of LEF without any specific regulatory mechanism, because the oxidation of PQH<sub>2</sub> is the slowest step in the photosynthetic electron transport system, and the amount of Cyt *b<sub>6</sub>/f* is normally smaller than those of PSII and PSI in plant leaves (Stiehl and Witt, 1969; Anderson, 1992; Kirchhoff et al., 2000; Yamori et al., 2010; Yamori et al., 2011; Schöttler and Tóth, 2014; Schöttler et al., 2015). Additionally, at present times  $\Delta\text{pH}$  across the thylakoid membrane is believed to regulate the oxidation activity of PQH<sub>2</sub> in Cyt *b<sub>6</sub>/f* for the induction of P700 oxidation in response to fluctuating environments in plant leaves (Kanazawa and Kramer, 2002; Kramer et al., 2004; Avenson et al., 2005; Nandha et al., 2007; Takizawa et al., 2007; Takizawa et al., 2008; Rott et al., 2011; Armbruster et al., 2014; Takagi et al., 2017). The possible function of RISE in plant leaves waits to be elucidated in future works.

## 9. Materials and Methods

### Plant materials

A male accession of *M. polymorpha*, Takaragaike (Tak)-1 was asexually maintained, according to previously described methods (Ishizaki et al., 2008). Plants were incubated on one-half-strength Gamborg's B5 agar medium (Gamborg et al., 1968) under a light–dark cycle (14 h-light, 22 °C, 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , white fluorescent lamp/10 h-dark, 20 °C). For biochemical and physiological measurements of plants, 2-week-old gemmalings were transferred from B5 agar medium onto moist vermiculite.

### Cyanobacterial and algal materials

Cyanobacterial cultures were maintained under continuous fluorescent lighting (25°C, 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) on BG-11 solid media (for S. 6803 and S. 7942) and A<sup>+</sup> solid media (for S. 7002) (Allen, 1968; Stevens and Porter, 1980). Cells from both cultures were inoculated into liquid media (initial OD<sub>750</sub>: 0.1–0.2) and grown on a rotary shaker (100 rpm) under continuous fluorescent lighting (25°C, 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at 20,000 (high) or 400 (ambient) ppm [CO<sub>2</sub>]. Optical densities of the media at 750 nm were measured with a spectrophotometer (U-2800A, Hitachi, Tokyo, Japan). For all photosynthetic parameter measurements, cells from the exponential growth phase were used.

The culture of the green alga *C. reinhardtii* was kept under continuous light conditions (25 °C, 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , fluorescent lamp) on tris-acetate-phosphate agar plates. Cells were inoculated into the liquid media, and was photoautotrophically grown in a liquid high-salt medium (Sueoka, 1960) on a rotary shaker (100 rpm) under the light–dark conditions described

above, at 20,000 ppm or ambient CO<sub>2</sub>. For all measurements, cells from the exponential growth phase were used.

The euglenoid *E. gracilis* Z (NIES-48) was photoautotrophically cultured in Cramer-Myers medium (Cramer and Myers, 1952) under light:dark conditions (25 °C, 16 h, 150 μmol photons m<sup>-2</sup> s<sup>-1</sup>, fluorescent lamp: 23 °C, 8 h, dark).

The diatom *P. tricornutum* (UTEX642) was photoautotrophically cultured in the artificial seawater medium described previously, with the addition of 0.31% half-strength Guillard's 'F' solution (Guillard and Ryther, 1962; Guillard, 1975) under light:dark conditions (22 °C, 14 h, 100 μmol photons m<sup>-2</sup> s<sup>-1</sup>, fluorescent lamp: 20 °C, 10 h, dark).

### **Determination of Chl**

The contents of Chl *a* and *b* in the *M. polymorpha* thalli were spectrophotometrically measured using a U-2800A spectrophotometer (Hitachi, Tokyo, Japan). For the measurement, extracts of thalli were obtained through incubation in 100% (v/v) *N,N*-dimethylformamide overnight. Both the Chl *a* and *b* contents in each extract were determined using the methods previously reported (Porra et al., 1989).

For Chl *a* measurement in cyanobacterial cells, the cells from 0.1–1.0 mL cultures were centrifugally harvested and resuspended by vortexing in 1 mL 100% (v/v) methanol. After incubation at room temperature for 5 min, the suspension was centrifuged at 10,000 × *g* for 5 min. Total Chl *a* was spectrophotometrically determined from the supernatant (Grimme and Boardman, 1972).

Both Chl *a* and Chl *b* in *C. reinhardtii* and *E. gracilis* were quantified as in the cyanobacterial cells following the above-mentioned method (Grimme and Boardman, 1972).

In the case of the diatom *P. tricornutum*, both Chl *a* and Chl *c* were spectroscopically

quantified using an equation described in the previous report (Jeffrey and Humphrey, 1975) in a 1 mL mixture containing 10% (v/v) distilled water, 10% (v/v) dimethyl sulfoxide, and 80% (v/v) acetone after the cells were centrifugally harvested.

### **Measurement of nitrogen**

To measure nitrogen, plant, cyanobacteria, and algae samples were dried overnight at 60 °C and then digested *via* Kjeldahl digestion with sulfuric acid. Total nitrogen contents were determined with Nessler's reagent after adding sodium potassium tartrate (Shimakawa et al., 2014).

### **O<sub>2</sub>-exchange measurement in a gas phase**

In a gas phase, O<sub>2</sub> was monitored simultaneously with Chl fluorescence. Plant leaves or liverwort thalli (2–10 cm<sup>2</sup>) were set in the O<sub>2</sub>-electrode chamber (LD2/3; Hansatech Ltd, King's Lynn, UK), and the Chl fluorescence was monitored using a Junior-PAM Chl fluorometer (Walz, Effeltrich, Germany) through a light-guided plastic-fiber set into the O<sub>2</sub>-electrode chamber (Hanawa et al., 2017). The temperature of the chamber was set to 25°C. Red AL was illuminated from the top of the O<sub>2</sub>-electrode chamber, and PFD were adjusted to the values indicated in the corresponding figure legends. Since the O<sub>2</sub>-electrode chamber was a closed system, CO<sub>2</sub>-saturated conditions were simulated by placing a fabric mat wetted with 1 M NaHCO<sub>3</sub> solution below the intact thalli to supply CO<sub>2</sub> at a concentration of approximately 1%. The photosynthetic parameters in PSII were calculated using Chl fluorescence parameters as described below.

### **O<sub>2</sub>-exchange measurement in a liquid phase**

In a liquid phase, net uptake and evolution of O<sub>2</sub> was measured simultaneously with Chl fluorescence. Cell samples in a reaction mixture or freshly prepared media (2 mL, 10–30 µg Chl *a* mL<sup>-1</sup>, see the figure legends) were stirred with a magnetic microstirrer and illuminated with white or red AL at 25°C except for the diatom *P. tricornutum* (20°C). A halogen lamp (Xenophot HLX 64625, Osram, München, Germany) from the LS2 light source (Hansatech, King's Lynn, UK) was used as the white AL source. Red AL was provided by the LS2 light source with filter sets in the range from 620 to 695 nm. O<sub>2</sub> was monitored continuously using an O<sub>2</sub> electrode (Hansatech, King's Lynn, UK) while the measuring cuvette remained open to allow diffusion of O<sub>2</sub> and CO<sub>2</sub> between the medium and the air (Hayashi et al., 2014; Shimakawa et al., 2015). The top of the cuvette was temporarily closed (1–3 min) while the O<sub>2</sub> evolution rate was determined.

### Measurement of Chl fluorescence

Fluorescence originating Chl *a* was measured using a Dual-PAM-100 fluorometer (Walz, Effeltrich, Germany), a Junior-PAM Chl fluorometer (Walz, Effeltrich, Germany), or a PAM-101 Chl fluorometer (Walz, Effeltrich, Germany) at 25 ± 2 °C. The photosynthetic parameters in PSII were calculated using Chl fluorescence parameters, as follows (Schreiber et al., 1986; Genty et al., 1989; van Kooten and Snel, 1990; Schreiber et al., 1995; Schreiber et al., 1995; Baker, 2008):  $F_v/F_m = (F_m - F_o)/F_m$ ;  $Y(II) = (F_m' - F_s')/F_m'$ ;  $NPQ = (F_m - F_m')/F_m'$ ;  $qP = (F_m' - F')/(F_m' - F_o)$ ;  $F_o$ , minimum fluorescence from a dark-adapted leaf;  $F_m$ , maximum fluorescence from a dark-adapted leaf;  $F_m'$ , maximum fluorescence from a light-adapted leaf; and  $F_s'$ , fluorescence emission from a light-adapted leaf. Pulse-amplitude modulated measuring light (0.1–0.5 µmol photons m<sup>-2</sup> s<sup>-1</sup>) was applied to determine  $F_o$ . Short-saturation pulses (10,000

$\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 300–1,000 ms) were applied to determine  $F_m$  and  $F_m'$ . Only for cyanobacteria,  $F_m$  was determined in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (final 5  $\mu\text{M}$ ) to exclude effects of state transition (Campbell et al., 1998).

During analysis with an Imaging-PAM (M-Series, Walz, Effeltrich, Germany), pulse-modulated excitation, actinic illumination, and saturation pulses were achieved with a blue LED lamp with a peak emission of 450 nm. Images of the fluorescence parameters were displayed with the help of a false color code ranging from black (0.0) through red, yellow, green, blue, and pink (1.0) (Singh et al., 2013).

### **Measurement of P700**

The photosynthetic parameters in PSI were calculated from the redox state of P700, which was measured with a Dual-PAM-100 fluorometer (Walz, Effeltrich, Germany) at  $25 \pm 2$  °C, as follows (Klughammer and Schreiber, 1994, 2008; Schreiber and Klughammer, 2008):  $Y(I) = (P_m' - P)/P_m$ ; quantum yield of non-photochemical energy dissipation due to the acceptor-side limitation,  $Y(NA) = (P_m - P_m')/P_m$ ; quantum yield of non-photochemical energy dissipation due to donor-side limitation,  $Y(ND) = P/P_m$ ;  $P_m$ , total amount of photo-oxidizable P700;  $P_m'$ , maximum amount of photo-oxidized P700 by a saturation pulse; and  $P$ , amount of photo-oxidized P700 at steady-state. Red AL was used to measure the photosynthetic parameters at PFD, as indicated in the corresponding figure legends. For the determination of  $P_m'$ , a short-saturation pulse ( $10,000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 300 ms) was applied and, for the determination of  $P_m$ , the short-saturation pulse was applied after 10 s illumination with a far red light (740 nm). To measure the absorbance of P700 in a liquid phase, the stirrer was turned off 5 s before SP was applied.

## Measurement of ECS

The signal of ECS was measured using a Dual-PAM-100 fluorometer equipped with a P515-analysis module (Klughammer et al., 2013). In this measurement, we used a 3010 DUAL gas exchange leaf chamber (Walz, Effeltrich, Germany). Ambient air was saturated with water vapor at  $17 \pm 2^\circ\text{C}$ , and the leaf temperature was maintained at  $25^\circ\text{C}$ . The ECSt,  $g_{\text{H}^+}$  in ATP synthase, and  $V_{\text{H}^+}$  were measured through dark-interval relaxation kinetics (DIRK) analysis, as described by the preceding reports (Sacksteder and Kramer, 2000; Cruz et al., 2001; Joliot and Joliot, 2002; Baker et al., 2007). For the DIRK analysis, we set the transient dark (600 ms) during illumination with AL. We measured the extent of the change in ECS as ECSt, and the half-life ( $\tau_{1/2}$ ) of ECS decay for the calculation of  $g_{\text{H}^+}$  ( $\text{s}^{-1}$ ). During the transient dark,  $V_{\text{H}^+}$  was estimated from the initial decay of ECS. The values of ECSt were normalized by dividing the magnitude of ECS decay in DIRK analysis by the magnitude of ECS induced by a  $10\ \mu\text{s}$ -single turnover flash. In separate experiments, ECSt was divided into  $\Delta\text{pH}$  and  $\Delta\Psi$  following the methods of Klughammer et al. (2013).

## Preparation of an anaerobic condition in a liquid phase

To measure the dependence of relative ETR on  $\text{O}_2$  in the “Part I–III”, we added glucose (5 mM), catalase ( $250\ \text{units mL}^{-1}$ , Wako, from bovine liver), and GlcOX ( $5\ \text{units mL}^{-1}$ , Wako, from *Aspergillus niger*) to the reaction mixture with the  $\text{O}_2$  electrode chamber closed to block the diffusion of air to the mixture. After photosynthetic  $\text{O}_2$  evolution rates decreased to 0, we added these agents and evaluated the relative ETR.

## Measurement of peroxidase activity

In the “Part II”, the activity of the Mehler-ascorbate peroxidase pathway (i.e., the water-water cycle) in cyanobacterial and algal cells was estimated from H<sub>2</sub>O<sub>2</sub>-dependent O<sub>2</sub> evolution rates during CO<sub>2</sub>-limited photosynthesis (Miyake et al., 1991). To exclude the effects of catalase, we added HA to the reaction medium at 0.1 mM (for *S. 7942* and *P. tricornutum*) or 0.5 mM (for *E. gracilis*).

### **Fluorescence emission spectra at 77 K**

With a micropipette, cyanobacterial and algal cells (100 µL) were sampled from the reaction mixture into the O<sub>2</sub> electrode chamber. These cells were subsequently injected into glass rod tubes that were plunged into liquid nitrogen within 3 s of sampling (Hayashi et al., 2014). Fluorescence emission spectra at 77 K were measured with a spectrofluorometer equipped with an integrating sphere (Jasco FP-6600/ILFC-543L). Cyanobacterial cells were excited at 600 nm, where phycobilisomes are mostly excited, and algal cells were excited at 480 nm, where light-harvesting chlorophyll complexes are generally excited.

### **Generation of mutants in cyanobacteria**

The triple mutant of *S. 6803* deficient in *flv1* (*sll1521*), *flv3* (*sll0550*), and *flv4* (*sll0217*) was generated by transforming  $\Delta flv1/3$  (Hayashi et al., 2014) using the *flv4* construct (Shimakawa et al., 2015). PCR (polymerase chain reaction) was used to confirm the complete segregation of *flv1* and *flv4* (Fig. 34A). The disruption of FLV3 proteins was verified by immunoblotting with a specific antibody to FLV3 as described below since a non-specific band was observed near the target band in the PCR analysis.

To construct the double mutant of *S. 7002* lacking *flvI* (*SYNPCC7002\_A1743*) and *flv3* (*SYNPCC7002\_A1321*) orthologs, PCR was used to amplify each genomic region encoding *A1743* and *A1321* with the up f and dn r primer sets (Table 9). They were then cloned into the pGEM-T Easy vector (Promega, Tokyo, Japan). The recombinant plasmids containing *A1743* and *A1321* were linearized and amplified by inverse PCR with the up f and dn r primer sets (Table 9). They were then applied to the In-Fusion reaction (Takara, Shiga, Japan) using chloramphenicol- and kanamycin-resistance genes (*Cm<sup>r</sup>* and *Kan<sup>r</sup>*) derived from pACYC184 and pUC4K vectors, respectively (Rose, 1988; Taylor and Rose, 1988). Transformations of *S. 7002* were performed by the standard procedure (Frigaard et al., 2004). Single mutants ( $\Delta flvI$  and  $\Delta flv3$ ) were selected on 0.5% BG-11 agar plates containing chloramphenicol (15  $\mu\text{g mL}^{-1}$ ) or kanamycin (50  $\mu\text{g mL}^{-1}$ ). The double mutant ( $\Delta flvI/3$ ) was generated by transforming  $\Delta flvI$  with the  $\Delta flv3$  construct. The mutants were selected on plates containing both chloramphenicol (15  $\mu\text{g mL}^{-1}$ ) and kanamycin (50  $\mu\text{g mL}^{-1}$ ). Complete segregation was confirmed by PCR (Fig. 31).

**Table 9.** Primers used for generating the mutants in *S. 7002*

| Name              | Sequences (5'-3')                        |
|-------------------|------------------------------------------|
| <i>A1743</i> up f | CTGGGATTCGAAGCACATTTT                    |
| <i>A1743</i> up r | TCTTACGTGCCGATCCGATCCAGTGGCGGTAGTTAT     |
| <i>A1743</i> dn f | TGACCGTGTGCTTCTACGCTGACCCAGAGGAAATTA     |
| <i>A1743</i> dn r | GCATAGATCACCCAATGGTCA                    |
| <i>A1321</i> up f | ATCCAGACAGAAAAGGTTAACGAC                 |
| <i>A1321</i> up r | AAACCGCCCAGTCTACCATAATCAGAGACGTAAAATACCG |
| <i>A1321</i> dn f | GTTGGGCTTCGGAATGGAGTTGCCGTAGAAATGGTC     |

### Generation of mutants in the liverwort *M. polymorpha*

To generate the *MpFlv1*-targeting vector, pJHY-TMp1 was used (Ishizaki et al., 2013). The 5' and 3' homology arms (4.6 and 4.5 kbp, respectively) were amplified from genomic DNA extracted from Tak-1 thalli through PCR using KOD Fx Neo (Toyobo, Osaka, Japan), with the primer sets T<sub>up</sub> and T<sub>dn</sub> used for the 5' and 3' homology arms, respectively (Table 10). The PCR products of these homology arms were cloned into the *PacI* and *AscI* sites of pJHY-TMp1 using the In-Fusion HD Cloning Kit (Takara, Shiga, Japan). Introduction of the targeting construct into *M. polymorpha* was performed using *Rhizobium radiobacter* C58C1 GV2260, as has been previously described (Ishizaki et al., 2008; Ishizaki et al., 2013). F<sub>1</sub> spores generated by crossing Tak-1 and Tak-2 were used for transformation. Isogenic lines (designated as G<sub>1</sub> lines) were obtained by isolating gemmae, which develop from single cells, and were screened for gene-targeted lines to use as  $\Delta MpFlv1$  by genotyping using a previously described method (Ishizaki et al., 2013) (Fig. 44B).

To generate complementation lines of  $\Delta MpFlv1$ , a binary vector, pMpGWB306, harboring a mutated acetolactate synthase gene that confers chlorosulfuron resistance was used (Ishizaki et al., 2015). The coding region of *MpFlv1* was amplified from cDNA from Tak-1 through PCR using KOD plus Neo (Toyobo, Osaka, Japan) with the primer set C (Table 10), and was then cloned into pENTR/D-TOPO (Thermo Fisher Scientific, Waltham, USA). The resultant *MpFlv1* cassette was cloned into pMpGWB306 using LR clonase II (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. The inserted *MpFlv1* was

driven by the CaMV 35S promoter (Ishizaki et al., 2015). Complementation lines were generated by transforming the resulting binary plasmids into regenerating thalli of  $\Delta MpFlv1$ , as has been previously described (Kubota et al., 2013). Several transformants were obtained through selection with chlorosulfuron and used as *cMpFlv1* lines.

### Measurement of transcripts

To check the gene expression of *MpFlv1*, reverse transcription-PCR was performed using KOD Fx Neo (Toyobo, Osaka, Japan) with cDNA from Tak-1,  $\Delta MpFlv1$ , and *cMpFlv1*. We used the *Actin* gene (*Mapoly0016s0137*) as a reference gene. The primer sets used (RT<sub>FLV</sub> and RT<sub>ACT</sub>) are listed in Table 10.

**Table 10.** Primers used for generating the mutants in *M. polymorpha*

| Name              | Sequences (5'-3')                            |
|-------------------|----------------------------------------------|
| T <sub>up</sub> F | CTAAGGTAGCGATTATCAAAGATGCATAGTACAACAAAAGCAGA |
| T <sub>up</sub> R | GCCCGGGCAAGCTTAGATCAAGGGTTTCGGGTCTCG         |
| T <sub>dn</sub> F | TAACTAGTGGCGCGGGCCTGCAGAGGCAGAAAAGAC         |
| T <sub>dn</sub> R | TTATCCCTAGGCGCGATGTGCAACAACGGGGGAAAGT        |
| A                 | GGCGTAAACCTGCCTCCTTCA                        |
| B                 | TGAGACGTACCACCTGCCAAGC                       |
| C                 | CAAAAAGTCAATGAACTGTCTGCAATCAA                |
| D                 | CGGGCGATGTGCTTGATCTT                         |
| C F               | CACCATGATGGCCCTTACGATGCC                     |
| C R               | GTAGCGTGCTCCTGTCTTCC                         |

|                     |                                |
|---------------------|--------------------------------|
| RT <sub>FLV</sub> F | GAATCACTCCCAAGGCGTTC           |
| RT <sub>FLV</sub> R | GAGGACGCTGTATGGACAGGC          |
| RT <sub>ACT</sub> F | GAGCGCGGTTACTCTTTCAC           |
| RT <sub>ACT</sub> R | GACCGTCAGGAAGCTCGTAG           |
| X                   | GAAGGCTTCTGATTGAAGTTTCCTTTTCTG |
| Y3                  | TGGATTGAACTTCTTTCGTATGGA       |

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### Immunoblot analysis

To check the deficit of FLV3 in the mutant  $\Delta flv1/3/4$  of S. 6803, the cell cultures (10 mL) were harvested by centrifugation and pellet resuspension in 500  $\mu$ L extraction buffer containing 50 mM HEPES-KOH (pH 7.5), 1 mM MgCl<sub>2</sub>, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The suspensions were homogenized with glass beads using Bug Crasher GM-01 (Taitec Co., Aichi, Japan) and centrifuged at  $13,000 \times g$  for 30 min at 4°C. The supernatants were treated as extracted soluble fractions. Protein concentrations in them were determined with the Pierce 660 nm Protein Assay (Thermo Scientific, Rockford, U.S.A.) using bovine serum albumin as the standard. Soluble fractions containing 5  $\mu$ g protein were analyzed by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to a polyvinylidene fluoride membrane and detected by an FLV3-specific antibody (kindly provided by Dr. H. Yamamoto, Kyoto University, Japan).

### Bioinformatics

All the S. 7002, S. 6803, and S. 7942 gene sequence data used in the “Part III” were obtained from cyanobase (<http://genome.microbedb.jp/CyanoBase>) (Fujisawa et al., 2014; Fujisawa et al., 2017). For the *flv1-4*, *cox*, *cyd*, *arto*, *ndhD1/2*, and *pgr5* gene sequences, BLAST searches were conducted in cyanobase.

### **Statistical analysis**

Student’s *t*-tests were applied to detect differences. All statistical analyses were performed using Microsoft Excel 2010 (Microsoft, Washington, U.S.A.), JMP8 (SAS Institute Inc., Tokyo, Japan), and Origin Pro (Lightstone, Tokyo, Japan).

## 10. Acknowledgements

The works in this doctoral dissertation were supported by the Japan Society for the Promotion of Science (JSPS, Scientific Research Grant No. 26450079 to C.M.). Additionally, I was supported as a JSPS research fellow (Scientific Research Grant No. 16J03443).

In the studies in this doctoral dissertation, we thank Prof. Akihiko Kondo, Prof. Tomohisa Hasunuma, and Dr. Shimpei Aikawa (Kobe University) for supplying the *Synechococcus* sp. PCC 7002 wild-type (in the “Part I and III”). We also thank Dr. Hiroshi Ohkawa (Hirosaki University) and Prof. Kintake Sonoike (Waseda University) for giving us the mutant  $\Delta ndhD1/2$  (in the “Part III”). Further, we thank Dr. Hiroshi Yamamoto (Kyoto University) for the kind gift of the anti-FLV3 antibody (in the “Part III”).

For writing this dissertation, I would like to thank Editage ([www.editage.jp](http://www.editage.jp)) and NAI, Inc. (<http://www.nai.co.jp/>) for English language editing.

Finally, I would like to thank all colleagues that helped me to develop the doctoral research.

## 11. Achievements

*In Chikahiro Miyake's laboratory (Kobe University) from October, 2011 to January, 2018.*

### Original Paper (Corresponding Author):

1. Ginga Shimakawa, Seiji Akimoto, Yoshifumi Ueno, Ayumi Wada, Keiichiro Shaku, Yuichiro Takahashi, Chikahiro Miyake (2016) Diversity in photosynthetic electron transport under [CO<sub>2</sub>]-limitation: the cyanobacterium *Synechococcus* sp. PCC 7002 and green alga *Chlamydomonas reinhardtii* drive an O<sub>2</sub>-dependent alternative electron flow and non-photochemical quenching of chlorophyll fluorescence during CO<sub>2</sub>-limited photosynthesis. *Photosynthesis Research* 130(1) 293–305.

### Original Paper (First Author):

1. Ginga Shimakawa, Tatsuya Iwamoto, Tomohito Mabuchi, Ryota Saito, Hiroshi Yamamoto, Katsumi Amako, Toshio Sugimoto, Amane Makino, Chikahiro Miyake (2013) Acrolein, an  $\alpha,\beta$ -unsaturated carbonyl, inhibits both growth and PSII activity in the cyanobacterium *Synechocystis* sp. PCC 6803. *Bioscience, Biotechnology, and Biochemistry* 77(8) 1655–1660.
2. Ginga Shimakawa\*, Mayumi Suzuki\*, Eriko Yamamoto, Akiko Nishi, Ryota Saito, Katsuhiko Sakamoto, Hiroshi Yamamoto, Amane Makino, Chikahiro Miyake (2013) Scavenging systems for reactive carbonyls in the cyanobacterium *Synechocystis* sp. PCC

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**Original Paper (Co-Author):**

1. Ryota Saito, Ginga Shimakawa, Akiko Nishi, Tatsuya Iwamoto, Katsuhiko Sakamoto, Hiroshi Yamamoto, Katsumi Amako, Amane Makino, Chikahiro Miyake (2013) Functional analysis of the AKR4C subfamily of *Arabidopsis thaliana*: Model structures, substrate specificity, acrolein toxicity, and responses to light and [CO<sub>2</sub>]. *Bioscience, Biotechnology, and Biochemistry* 77(10) 2038–2045.

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3. Tomohisa Hasunuma, Mami Matsuda, Youhei Senga, Shimpei Aikawa, Masakazu Toyoshima, Ginga Shimakawa, Chikahiro Miyake, Akihiko Kondo (2014) Overexpression of *flv3* improves photosynthesis in the cyanobacterium *Synechocystis* sp. PCC6803 by enhancement of alternative electron flow. *Biotechnology for Biofuels* 7: 493.
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5. Daisuke Takagi, Kimitsune Ishizaki, Hitomi Hanawa, Tomohito Mabuchi, Ginga Shimakawa, Hiroshi Yamamoto, Chikahiro Miyake (2017) Diversity of strategies for escaping reactive oxygen species production within photosystem I among land plants. *Physiologia Plantarum* 161(1) 56–74.
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**Review:**

1. Ginga Shimakawa, Mayumi Suzuki, Eriko Yamamoto, Ryota Saito, Tatsuya Iwamoto, Akiko Nishi, Chikahiro Miyake (2014) Why don't plants have diabetes? Systems for scavenging reactive carbonyls in photosynthetic organisms. *Biochemical Society Transactions* 42(2) 543–547. *World Biomedical Frontiers* ISSN: 2328-0166.
2. Ginga Shimakawa, Chikahiro Miyake (2014) The mechanisms of production and detoxification of dicarbonyls in photosynthetic organisms. *IMARS Highlights* 9(3) 5–13.
3. 嶋川 銀河 (2015) シアノバクテリアの光合成における酸素利用. 光合成研究 25(1) 16–21.
4. 嶋川 銀河, 三宅 親弘 (2017) 植物が安心して光合成できるワケ～PSIを光傷害から護る P700 酸化システム～. 光合成研究 27(1) 4–15.

**Presentation (Invited lecture):**

1. Ginga Shimakawa, Chikahiro Miyake (2015) Flavodiiron 2 and 4 proteins mediate an O<sub>2</sub>-dependent alternative electron flow in *Synechocystis* sp. PCC 6803 under CO<sub>2</sub>-limited conditions. (Oral) International Meeting Photosynthesis Research for Sustainability – 2015

*in honor of George Papageorgiou, Crete, Greece.*

2. 嶋川 銀河, 三宅 親弘 (2016) 藍藻における P700 酸化メカニズムの多様性～なぜ P700 は酸化される必要があるのか?～ (Oral) 光合成学会若手の会セミナー, 東京.
3. Ginga Shimakawa, Kimitsune Ishizaki, Moeko Tanaka, Chikahiro Miyake (2016) Photosynthetic electron and proton transport in the evolutionary bridge from algae to plants: Do flavodiiron proteins function in photoprotection in the liverwort *Marchantia polymorpha*? (Oral) Photosynthetic Electron and Proton Transport in Plants and Algae; operation, regulation and function, Arnhem, the Netherlands.

**Presentation (Speaker):**

1. Ginga Shimakawa, Hiroshi Yamamoto, Amane Makino, Chikahiro Miyake (2013) Suppression of respiration retard induction of photosynthesis in the cyanobacterium *Synechocystis* sp. PCC 6803. (Poster) The 16th International Congress on Photosynthesis Research, St. Louis, USA.
2. 嶋川 銀河, 鈴木 麻由実, 三宅 親弘 (2013) Scavenging systems for reactive carbonyls in cyanobacterium *Synechocystis* sp. PCC 6803. (Oral and Poster) 植物生態学・分子生理学コンソーシアムによる陸上植物の高 CO<sub>2</sub> 応答の包括的解明 第 4 回若手ワークショップ, 宮城.

3. Ginga Shimakawa, Chikahiro Miyake (2013) Production and detoxification mechanisms of methylglyoxal in photosynthetic organisms. (Oral and Poster) Glyoxalase Centennial: 100 Years of Glyoxalase Research and Emergence of Dicarbonyl Stress, Coventry, UK.
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7. 嶋川 銀河 (2014) P700 解析の原理とデータの解釈. (Oral) PAM を用いた光合成解析技術セミナー, 東京.
8. Ginga Shimakawa, Kimitsune Ishizaki, Shigeyuki Tsukamoto, Chikahiro Miyake (2014) Glyoxalase system in a liverwort *Marchantia polymorpha*: A defense system against plant diabetes. (Poster) Marchantia Workshop 2014, Kobe, Japan.
9. 嶋川 銀河, 釋 啓一郎, 三宅 親弘 (2015) Flavodiiron proteins 2 and 4 drive and O<sub>2</sub>-dependent alternative electron flow to replace photosynthetic linear electron flow in

*Synechocystis* sp. PCC 6803. (Oral) 第 56 回 日本植物生理学会年会, 東京.

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11. 嶋川 銀河, 三宅 親弘 (2015) 生理解析から見るラン藻のエレクトロン・シンクの多様性. (Oral) 平成 27 年度 ラン藻ゲノム交流会, 東京.
12. 嶋川 銀河, 三宅 親弘 (2015) シアノバクテリアにおける Alternative electron sink の生理活性と分子メカニズム. (Oral) 平成 27 年度 植物科学談話会, 大阪.
13. 嶋川 銀河, 三宅 親弘 (2015) 藍藻において暗呼吸は迅速な光合成誘導に不可欠である. (Poster) 藍藻の分子生物学 2015, 千葉.
14. 嶋川 銀河, 松田 祐介, 中島 健介, 三宅 親弘 (2015) Diversity of the response of photosynthetic electron transport to low-[CO<sub>2</sub>]: O<sub>2</sub> uses and the use-nots. (Oral) 第 2 回 分子珪藻研究会, 大阪.
15. 嶋川 銀河, 和田 亜祐美, 釋 啓一郎, 秋本 誠志, 植野 嘉文, 松田 祐介, 中島 健介, 高橋 裕一郎, 田茂井 政宏, 重岡 成, 三宅 親弘 (2016) 光合成電子伝達反応の低 CO<sub>2</sub> 応答の多様性: O<sub>2</sub> を使う者と使わざる者. (Oral) 第 57 回 日本植物生理学会年会, 岩手.
16. Ginga Shimakawa, Chikahiro Miyake (2016) Oxidation of P700 in PSI is essential for the growth of cyanobacteria. (Poster) The 17th International Congress on Photosynthesis

Research, Maastricht, the Netherlands.

17. 嶋川 銀河, 三宅 親弘 (2016) 光化学系 I 防御戦略の多様性～なぜ光合成生物は安全に光合成できるのか?～ (Oral) 第 3 回 分子珪藻研究会, 兵庫.
18. 嶋川 銀河, 石崎 公庸, 田中 萌子, 高木 大輔, 塙 仁美, 三宅 親弘 (2017) 基部陸上植物における PSI 防御戦略: ゼニゴケにおいて Flavodiiron タンパク質は P700 酸化に働く. (Oral) 第 58 回 日本植物生理学会年会, 鹿児島.
19. Ginga Shimakawa, Yusuke Matsuda, Chikahiro Miyake (2017) Universality and diversity of P700 oxidation for the protection of photosystem I against ROS-attacks in photosynthetic organisms: *Phaeodactylum tricornutum* oxidizes P700 without O<sub>2</sub>-usage. (Oral) The IVth International Conference Molecular Life of Diatoms, Kobe, Japan.

#### Prize:

1. [Poster Prize] 嶋川 銀河, 釋 啓一郎, 三宅 親弘 (2014) ランソウ Flavodiiron protein による Alternative electron flow の機能解明とその活性評価. (Poster) 第 5 回 日本光合成学会年会, 奈良.
2. [六篠賞] 嶋川 銀河 (2015) 神戸大学農学研究科.
3. [第一種奨学金の全額返済免除] 嶋川 銀河 (2015) 日本学生支援機構.

4. [Young Talents Award] Ginga Shimakawa, Chikahiro Miyake (2015) Flavodiiron 2 and 4 proteins mediate an O<sub>2</sub>-dependent alternative electron flow in *Synechocystis* sp. PCC 6803 under CO<sub>2</sub>-limited conditions. (Oral) International Meeting Photosynthesis Research for Sustainability – 2015 *in honor of George Papageorgiou*, Crete, Greece.
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