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Review

Alternative Electron Flows (Water–Water Cycle and Cyclic Electron Flow Around PSI) in Photosynthesis: Molecular Mechanisms and Physiological Functions

Chikahiro Miyake*

Department of Biological and Environmental Science, Faculty of Agriculture, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai, Nada, Kobe, 657-8501 Japan

*Corresponding author: E-mail, cmiyake@hawk.kobe-u.ac.jp; Fax, +81-78-803-5851

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An electron flow in addition to the major electron sinks in C_3 plants [both photosynthetic carbon reduction (PCR) and photorespiratory carbon oxidation (PCO) cycles] is termed an alternative electron flow (AEF) and functions in the chloroplasts of leaves. The water–water cycle (WWC; Mehler–ascorbate peroxidase pathway) and cyclic electron flow around PSI (CEF-PSI) have been studied as the main AEFs in chloroplasts and are proposed to play a physiologically important role in both the regulation of photosynthesis and the alleviation of photoinhibition. In the present review, I discuss the molecular mechanisms of both AEFs and their functions in vivo. To determine their physiological function, accurate measurement of the electron flux of AEFs in vivo are required. Methods to assay electron flux in CEF-PSI have been developed recently and their problematic points are discussed. The common physiological function of both the WWC and CEF-PSI is the supply of ATP to drive net CO_2 assimilation. The requirement for ATP depends on the activities of both PCR and PCO cycles, and changes in both WWC and CEF-PSI were compared with the data obtained in intact leaves. Furthermore, the fact that CEF-PSI cannot function independently has been demonstrated. I propose a model for the regulation of CEF-PSI by WWC, in which WWC is indispensable as an electron sink for the expression of CEF-PSI activity.

Keywords: Alternative electron flow • Cyclic electron flow • Mehler–ascorbate peroxidase (MAP) pathway • Photosynthesis • Water–water cycle.

Abbreviations: AEF, alternative electron flow; APX, ascorbate peroxidase; Asc, ascorbate; CEF-PSI, cyclic electron flow around PSI; Ci, intercellular partial pressure of CO_2; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; Fd, ferredoxin; FNR, Fd-NADP oxidoreductase; FQR, Fd-quinone oxidoreductase; FTS, Fd–thioredoxin system; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione Jcef, electron flux in CEF; Jw, electron flux in WWC; Jg, electron flux in both PCR and PCO cycles; LEF, linear electron flow; MAP pathway, Mehler–ascorbate peroxidase pathway; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; NDH, NAD(P)H dehydrogenase; NPQ, non-photochemical quenching; PC, plastocyanin; PCO, photorespiratory carbon oxidation; PCR, photosynthetic carbon reduction; PET, photosynthetic electron transport; PQ, plastoquinone; qL, photochemical quenching; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate; SOD, superoxide dismutase; WWC, water–water cycle.

Introduction

During photosynthesis of plants, sunlight causes many kinds of electron transport. The light energy of the sun is converted to electron flows in the photosynthetic electron transport system in chloroplasts. Its energy is absorbed by the light-harvesting Chl molecules in both PSI and PSII, and is then used for the excitation of both the reaction center Chls. The photoexcitation of the PSII reaction center leads to the charge separation of the reaction center Chl P680, with both production of $P680^+$ and donation of the released electron to the plastoquinone (PQ) pool. $P680^+$ then oxidizes water with O_2 evolved. The photoexcitation of the PSI reaction center (P700) leads to its charge separation with both production of $P700^+$ and donation of the released electron to ferredoxin (Fd). $P700^+$ then oxidizes plastocyanin (PC). The photoreduction–oxidation cycles of both PSII and PSI reaction centers are linked, through which the Cyt b_6/f complex mediates the oxidation of the reduced PQ pool and reduction of the oxidized PC. As a result, the electron flow from water to Fd is completed, and this is termed the photosynthetic linear electron flow (LEF).

In LEF, acidification of the thylakoid lumen is induced by both the photooxidation of water in PSII and the oxidation of the reduced PQ by the Cyt b_6/f complex. Acidification results in a proton gradient across the thylakoid membranes (ΔpH)

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and produces free energy for the synthesis of ATP by ATP synthase. The accumulated electrons at the reducing side of PSI flow to several electron transport systems through Fd. In C_3 plants, the main electron sink reactions are both the photosynthetic carbon reduction (PCR) and the photorespiratory carbon oxidation (PCO) cycles, where NADPH produced by Fd-NADP oxidoreductase (FNR) and Fd photoreduced by PSI function as electron donors to reduce CO_2 to sugars.

The light energy absorbed by chloroplasts is used in metabolic pathways. The absolute requirement for a flow of light energy as an alternative to photosynthetic CO_2 assimilation can be recognized, for example, at least in the assimilation of nitrogen and sulfur because these nutrients are indispensable for plant growth. What is the significance of the existence of these other electron flow pathways?

Electron flow in pathways other than either PCR or PCO cycles in the photosynthetic electron transport system in chloroplasts is defined as alternative electron flow (AEF). The electron flows proposed as AEFs are as follows: (i) O_2-dependent electron flow, termed the Mehler–ascorbate peroxidase (MAP) pathway (Schreiber et al. 1995) or the the water–water cycle (WWC) (Asada 1999)); (ii) cyclic electron flow around PSI (CEF-PSI) (Heber 2002); (iii) cyclic electron flow around PSII (CEF-PSII) (Miyake et al. 2002); (iv) chlororespiration mediated by plastid terminal oxidase (PTOX) (Peltier et al. 2010); (v) the mitochondrial alternative oxidase pathway (Yoshida et al. 2008); and (vi) the pathways for mineral nutrient assimilation.

To elucidate the physiological significance of these AEFs in vivo, precise evaluation of each electron flux is required. In the present review, molecular mechanisms, measurement of the electron fluxes and the physiological responses to environmental stress of both the WWC and CEF-PSI, whose physiological functions are proposed in photosynthesis, are described. Furthermore, the problematic points still to be resolved are discussed.

The Water–Water Cycle (MAP Pathway)

Molecular mechanism of the WWC

Study of the WWC started with the discovery of light-dependent O_2 uptake in chloroplasts. In 1951, Mehler identified the function of O_2 as a Hill oxidant (Mehler 1951a, Mehler 1951b). Later, Furbank and Badger (1983) showed that O_2-dependent electron flow induced the production of ATP in thylakoid membranes, and Asada showed that the light-dependent O_2 uptake induced the univalent reduction of O_2 to superoxide anion radical (O_2^-) in chloroplasts (Asada 1974). The univalent reduction of O_2 was inhibited by DCMU, and O_2 accepted electrons at the reducing side of PSI as a Hill oxidant.

Several molecular species have been identified as the possible catalyst for O_2 photoreduction in chloroplasts: (i) the Fe/S center in the PSI complex (Asada and Takahashi 1987, Asada 1996, Asada 1999); (ii) Fd at PSI (Furbank and Badger 1983, Badger 1985); and (iii) flavoproteins in chloroplasts (Miyake et al. 1998). The FAD moiety in flavoprotein is photoreduced by PSI and the reduced FAD rapidly donates electrons to O_2, producing O_2^-. The Michaelis constant for O_2 photoreduction in both intact leaves and intact chloroplasts ranges from 60 to 80 μM (Marrsho et al. 1979, Furbank et al. 1982, Furbank and Badger 1983). The fact that there are several candidates for the photoreduction of O_2 suggests that the production of reactive oxygen species (ROS) at PSI has a physiological function in photosynthesis, as described below.

The superoxide anion radical produced by the photoreduction of O_2 at PSI is converted to H_2O_2 and O_2 by the enzyme superoxide dismutase (SOD). Furthermore, unless SOD functions, O_2^- spontaneously disproportionates to H_2O_2 and O_2, i.e. the photoreduction of O_2 in thylakoid membranes of chloroplasts is accompanied by the production of ROS (O_2^- and H_2O_2).

Both O_2^- and H_2O_2 oxidize chloroplast enzymes, listed below, which lose their activity. H_2O_2 reduces photosynthetic activity to half, even at the relatively low concentration of 10 μM (Kaiser 1976). This is due to the oxidative inactivation by H_2O_2 of Calvin cycle enzymes [fructose 1,6-bisphosphatase (FBPase), NADP-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribulose 5-phosphate kinase (PRK) and sedoheptulose 1,7-bisphosphatase (SBPase)] (Kaiser 1979, Tanaka et al. 1982). These enzymes have thiol groups in their amino acids. The thiol groups are oxidized by H_2O_2, resulting in the formation of disulfide bridges between the groups and leading to the inactive form. In illuminated chloroplasts, disulfide bridges in the inactivated enzymes are reduced to the thiol groups by the Fd–thioredoxin system (FTS), and the enzyme recovers activity (Leegood et al. 1985, Buchanan 1991). Therefore, the steady-state activities of these enzymes are determined by the balance between the H_2O_2-depedent oxidation rate and the FTS-dependent reduction rate. Thus, an increase in the accumulation rate of H_2O_2 in chloroplasts lowers photosynthetic activity and suppresses plant growth.

On the other hand, ROS also cause irreversible oxidative damage in chloroplasts. Accumulation of both O_2^- and H_2O_2 stimulates the production of hydroxyl radicals (·OH) by the transition metal-catalyzed Harber–Weiss reaction. ·OH has a redox potential higher than that of either O_2^- or H_2O_2, and can fragment DNA, proteins and lipids (Asada and Takahashi 1987). In particular, the stimulated production of ·OH in chloroplasts fragments both ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and glutamine synthase, and these enzymes oxidatively degrade (Fucci et al. 1983, Ishida et al. 1997, Ishida et al. 1998). Furthermore, at lower temperatures, ·OH decomposes the PSI complex in thylakoid membranes (Sonoike et al. 1996, Tjus et al. 2001). These oxidative attacks by ·OH also cause a reduction in photosynthesis activity. Furthermore, the accumulated H_2O_2 oxidatively degrades ascorbate peroxidase (APX) in chloroplasts (Miyake and Asada 1996), resulting in the stimulated accumulation of H_2O_2 and enhanced inactivation of photosynthesis (Miyake et al. 2006).

From observations of plants in the field, Asada was prompted to ask 'Why do plants not suffer sunburn?' and, from the intriguing fact that plants manage to photosynthesize under severe natural conditions, he proposed the hypothesis that plant chloroplasts have a protective mechanism against oxidative damage by ROS. During research to examine this hypothesis, SOD was identified in chloroplasts (Asada et al. 1973). Plants have three isoforms of SOD: Cu, Zn-SOD, Fe-SOD and Mn-SOD (Asada and Takahashi 1987). In chloroplasts, two types of SOD, Cu, Zn-SOD and Fe-SOD, are localized (Asada et al. 1973, Jackson et al. 1978). Thereafter, SODs are found in the vicinity of PSI and their concentration reaches about 1 mM (Ogawa et al. 1995, Asada 1996, Asada 1999).

Scavenging of O_2^- by SOD results in the production of H_2O_2 localized at the site of SOD itself. Unless H_2O_2 is removed, it accumulates and causes oxidative damage to chloroplast components, as described above. Asada's group identified the peroxidase that reduces H_2O_2 to H_2O using ascorbate (Asc) as the electron donor, APX, in the stroma fraction of chloroplasts (Nakano and Asada 1987; stroma-localized APX, sAPX). Subsequently, Asc peroxidase (APX) bound to thylakoid membranes (thylakoid-bound APX, tAPX) was identified (Miyake and Asada 1992, Miyake et al. 1993). The APX homolog, TL29, was found in the thylakoid lumen (Granlund et al. 2009); however, TL29 did not show any APX activity.

tAPX is localized in the vicinity of PSI in thylakoid membranes, where O_2 is photoreduced. The concentration of tAPX reaches about 1 mM (Asada 1996). The steady-state concentration of H_2O_2 in chloroplasts is estimated to be 30–40 nM from $Vp(H_2O_2)=k1[tAPX][H_2O_2]$, where $Vp(H_2O_2)$ is the production rate of H_2O_2 and is estimated to be 330–445 $\mu M\,s^{-1}$, $k1$ is $1.2\times10^7\,M^{-1}\,s^{-1}$ (Miyake and Asada 1992) and Asc is assumed to be >10 mM, which is the saturated concentration for the tAPX reaction. Chloroplasts contain Asc at a concentration of 10–30 mM, and tAPX lowers the steady-state concentration of H_2O_2 to about 1/100, at which concentration photosynthetic activity ceases.

In higher plants, APX proteins are localized in chloroplasts, cytosol, mitochondria and peroxisomes (Asada 1999). In contrast to chloroplast-localized APXs, other isoforms of APX do not suffer from inactivation by H_2O_2. This can be used to determine selectively the chloroplast-localized APX activity. Amako et al. (1994) developed a method to differentiate chloroplast APX activity from other APX activity by treating the extracts from intact leaves with H_2O_2, which inactivates chloroplast APX.

For continuous scavenging of H_2O_2 photoproduced in chloroplasts, the electron donor for APX, Asc, needs to be regenerated. The maximum production rate of H_2O_2 estimated in intact chloroplasts reaches about 450 $\mu M\,s^{-1}$. Unless Asc is regenerated, even at a concentration of about 30 mM in chloroplasts, it will be consumed within 60 s (Asada 1999). The existence of a regeneration system for Asc in chloroplasts was demonstrated by an elegant series of experiments (Nakano and Asada 1980, Nakano and Asada 1981, Asada and Badger 1984). In these, O_2 evolution following the repetitive addition of H_2O_2 to illuminated chloroplasts was measured. H_2O_2-dependent O_2 evolution was inhibited by KCN, the inhibitor of the APX reaction. However, the addition of H_2O_2 in the dark inhibited H_2O_2-dependent O_2 evolution in illuminated chloroplasts. These results indicate that light energy is required for the continuous scavenging of H_2O_2 by APX, i.e. Asc is regenerated under illuminated conditions. H_2O_2 added to the chloroplasts in the dark inactivated APX because all the Asc was consumed.

H_2O_2-dependent O_2 evolution in the illuminated chloroplasts shows the production of Hill oxidant by the APX reaction. Furthermore, it indicates that the APX reaction couples to the photosynthetic electron transport reaction (Asada 1999). Asada searched for the enzymes that regenerate Asc using NAD(P)H as electron donor and identified two enzymes for the regeneration of Asc in the stroma fraction of chloroplasts (Asada 1996, Asada 1999). The first is the monodehydroascorbate radical (MDA) reductase (MDAR) (Hossain and Asada 1985). MDAR reduces the primary oxidation product of Asc, MDA, produced in the APX reaction to Asc using NAD(P)H.

$$NAD(P)H + 2\,MDA \rightarrow NAD(P)^+ + 2\,Asc\ (MDAR\ reaction)$$

Second is dehydroascorbate (DHA) reductase (DHAR) (Hossain and Asada 1984). DHAR reduces DHA produced by the disproportionation of MDA to Asc using reduced glutathione (GSH) as electron donor.

$$2\,GSH + 2\,DHA \rightarrow GSSG + 2\,Asc\ (DHAR\ reaction)$$

The oxidized glutathione, GSSG, is reduced to GSH by the reaction of GSH reductase (GR) localized in the stroma fraction of chloroplasts. These reactions for Asc regeneration produce $NAD(P)^+$ as a Hill oxidant, i.e. coupling of O_2 photoreduction at PSI of thylakoid membranes with the regeneration of Asc in chloroplasts implies that ROS (O_2^- and H_2O_2) produced by the light energy is scavenged by the light energy itself.

These reactions for Asc regeneration proceed in the stroma fraction in chloroplasts. Subsequently, Miyake and Asada (1992) found that MDA is directly photoreduced to Asc by PSI of thylakoid membranes, and MDA functions as a Hill oxidant. Adding H_2O_2 to the illuminated thylakoid membranes enhances the quantum yield of PSII. The stimulation of the electron flux in PSII is inhibited by DCMU and dibromothymoquinone (DBMIB). These results indicate that MDA, produced by the thylakoid-bound APX reaction, is photoreduced by PSI of thylakoid membranes and Asc is regenerated. Furthermore, they elucidated that Fd enhances the photoreduction of MDA to Asc at PSI (Miyake and Asada 1994). These facts show that, in addition to the stroma scavenging system of H_2O_2, an H_2O_2-scavenging system functions on thylakoid membranes where thylakoid-bound APX rapidly scavenges H_2O_2 produced by the SOD reaction at PSI of thylakoids and then MDA produced by the APX reaction is photoreduced to Asc.

In summary, chloroplasts have two systems for scavenging H_2O_2. First is the stroma system, where MDAR, DHAR and GR

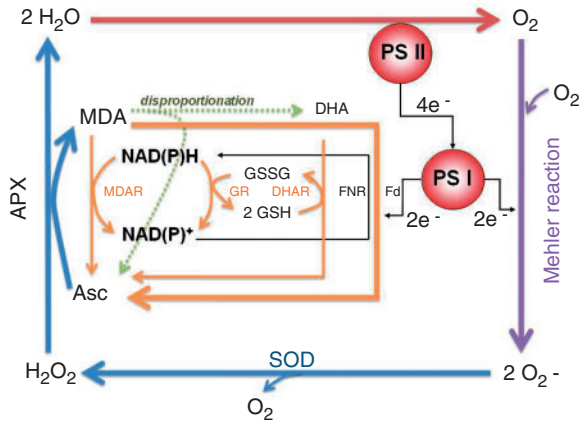

Fig. 1 The water–water cycle, which starts to turn over on the photoreduction of O_2 at PSI to superoxide radical (O_2^-) (Mehler reaction, purple arrow), with the concomitant evolution of O_2 by the photooxidation of H_2O at PSII (red arrow). The O_2^- is disproportionated by superoxide dismutase (SOD) to H_2O_2 and O_2 (blue arrow). The H_2O_2 is reduced to H_2O by ascorbate peroxidase (APX) (blue arrow). In the APX reaction, ascorbate (Asc) functions as the electron donor for APX, and Asc is univalently oxidized to the monodehydroascorbate radical (MDA). Asc is regenerated through three routes (yellow arrows and green arrows): first, MDA is directly reduced to Asc by ferredoxin (Fd); secondly, MDA is reduced to Asc by MDA reductase (MDAR). MDAR uses NAD(P)H as the electron donor; thirdly, MDA disproportionates to Asc and dehydroascorbate (DHA). DHA is reduced to Asc by DHA reductase (DHAR) (yellow arrow). DHAR uses GSH as the electron donor. GSH is regenerated by the reduction of GSSG by GSH reductase (GR) (yellow arrow). GR uses NAD(P)H as the electron donor. Both NAD(P)H and the reduced Fd are regenerated by Fd-NADP oxidoreductase (FNR) and the PSI of thylakoid membranes, respectively. These sequential and parallel reactions are termed the Mehler–ascorbate peroxidase (MAP) pathway, or the water–water cycle (WWC).

function for the regeneration of Asc, and the second is the thylakoid system, where Fd functions.

The O_2-dependent sequential reactions (production of ROS, scavenging of ROS and regeneration of Asc) in chloroplasts are illustrated in **Fig. 1**. As clearly shown, the regeneration reaction of Asc starts with the ROS production reaction, i.e. the regeneration of Asc depends on O_2 photoreduction. Furthermore, electron flow to both reaction systems originates from the photooxidation of H_2O in PSII and converges to H_2O. These O_2-dependent sequential electron flows were first termed the Mehler–ascorbate peroxidase (MAP) pathway (Neubauer and Schreiber 1989), and later given the name the water–water cycle (WWC) (Miyake et al. 1998, Asada 1999, Peltier et al. 2010).

Physiological function of the WWC

Physiological function of the MAP pathway, the WWC, are proposed as follows (Asada 1999, Ort and Baker 2002): (i) scavenging of ROS in chloroplasts; (ii) electron sink activity; (iii) dissipation of excess photon energy against photosynthesis as observed in the induction of non-photochemical quenching (NPQ) of Chl fluorescence; and (iv) the supply of ATP to support CO_2 assimilation in photosynthesis.

The first function is described above. However, the WWC does not scavenge ROS produced in chloroplasts perfectly. Although H_2O_2 added to illuminated chloroplasts is scavenged by APX, as shown by Nakano and Asada (1985), a prolonged illumination of chloroplasts under limited supply of CO_2 to the Calvin cycle causes chloroplasts to lose the scavenging activity of H_2O_2 in the WWC (Miyake et al. 2006). With longer illumination time, H_2O_2 starts to leak from chloroplasts, where APX is inactivated. These results indicate that an increase in the production rate of H_2O_2 intrinsically photoproduced at PSI decreases the reduction efficiency of compound I of APX by Asc, resulting in a higher probability of the oxidative degradation of compound I by H_2O_2 to the inactive form (Miyake and Asada 1996). Under natural conditions, does only the WWC function in chloroplasts as an electron sink? To answer this question, we have to find and analyze the activity of the WWC in vivo, as described below.

The second function of the WWC is its electron sink activity. Its ability is evaluated as an electron flux. The electron flux in the WWC of chloroplasts is estimated by the quantum yield of PSII [Φ(PSII)], which is obtained from analysis of Chl fluorescence (Schreiber and Neubauer 1990, Miyake et al. 2006). Three results provide evidence that Φ(PSII) in chloroplasts is driven by the WWC. First, under anaerobic conditions, Φ(PSII) is not observed (Schreiber and Nuebauer 1990, Hormann et al. 1994). Secondly, the inactivation of APX decreases Φ(PSII) by half (Miyake et al. 2006). Thirdly, in chloroplasts of *Galdieria partita*, tolerance of APX for H_2O_2 is different from that in higher plant APX, and H_2O_2 photoproduced with a limited supply of CO_2 to the Calvin cycle is scavenged, i.e. Φ(PSII) is maintained unless APX loses its activity (Miyake et al. 2006). These results indicate that the MAP pathway, the WWC, functions as the main electron sink under the suppressed condition of photosynthesis. Miyake et al. (2006) determined the required amount of APX for the support of electron sink ability of the WWC to be at least 40% of the total APX in chloroplasts. This estimate suggests that plants require about 2.5-fold the amount of APX indispensable for the WWC to drive CO_2 assimilation in natural conditions.

The electron sink ability of the WWC in vivo was demonstrated in algae by Radmer and Kok (1976). They observed light-dependent $^{18}O_2$ uptake by algal cells. The uptake rate of $^{18}O_2$ in the induction phase of photosynthesis equals the evolution rate of $^{16}O_2$. Furthermore, its rate decreases with the start of CO_2 assimilation, i.e. under the suppressed condition of photosynthesis, the WWC functions as an electron sink. Subsequently, Baker's group demonstrated the electron flux in the WWC in a C_4 plant, maize (Fryer et al. 1998). They analyzed Φ(PSII), simultaneously with the CO_2 exchange rate. Φ(PSII) indicates the electron source activity and the net CO_2 assimilation rate indicates the electron sink activity. In C_4 plants, the PCO cycle does not function. Therefore, four times

the CO_2 assimilation rate corresponds to the electron sink activity. They found that the electron source activity exceeds the sink activity under photosynthetic conditions, i.e. excess electron flux is driven by the WWC. Furthermore, Miyake and Yokota (2000), Laisk and Loreto (1996) and Laisk et al. (2007) reached the same conclusion using the same method in C_3 plants. Also, Biehler and Fock (1996) evaluated the WWC activity by analysis of the light-dependent $^{18}O_2$ uptake rate in C_3 plants. The electron sink ability of the WWC increases with the saturation of photosynthesis against light intensity. Electron flux in the WWC occupies about 10% of the total electron flux in PSII. Furthermore, lowering the partial pressure of CO_2 enhances WWC activity. Electron flux in the WWC occupies about 20% of the total electron flux in PSII. Makino's group showed the corresponding activity of the WWC to total electron flux in PSII at lower temperature in rice leaves (Hirotsu et al. 2004). These results suggest that WWC functions as an electron sink with a large electron flux.

Park et al. (1996) demonstrated that WWC alleviates photoinhibition of PSII by functioning as an electron sink. They compared the photoinhibition of PSII under several conditions of gas composition around leaves at high light intensity. Under atmospheric conditions (both 370 p.p.m. CO_2 and 20% O_2) where both PCR and PCO cycles and the WWC function, 20% O_2 where both the PCO cycle and the WWC function, and 2% O_2 where only the WWC functions, the PQ pool was oxidized and PSII photoinhibition was alleviated. On the other hand, anaerobic condition suppressed the electron flux in the WWC and shifted the redox level of the PQ pool to the reduced state, resulting in the enhancement of PSII photoinhibition (Park et al. 1996). Accumulation of electrons in the photosynthetic electron transport (PET) system limits the charge separation of the reaction center Chl in PSII, P680, and the excited P680 de-excites to the excited triplet state, ^{3}P680*. The triplet P680 transfers the energy to excite O_2 to the singlet O_2. The singlet O_2 oxidatively degrades proteins around P680 and lipid membranes, leading to inactivation of PSII (Asada 1996, Ruban 2009). The WWC suppresses the accumulation of electrons in the PET system by electron sink activity. The electron sink activity of the WWC in vivo can be observed as a higher value of the Chl fluorescence parameter, qL, which shows the redox level of the PQ pool (Kramer et al. 2004, Miyake et al. 2009). The oxidized PQ pool is reflected as a higher qL value.

The third physiological function of the WWC, the induction of NPQ of Chl fluorescence, is due to the formation of a ΔpH across thylakoid membranes by the turnover of the WWC (Schreiber and Neubauer 1990, Neubauer and Yamamoto 1992). The carotenoid pigment, zeaxanthin, which accepts the exciton from the excited triplet P680 and dissipates its energy safely as heat, is produced with the reaction catalyzed by violaxanthin de-epoxidase. Violaxanthin de-epoxidase is activated by acidification of the luminal side of thylakoid membranes. The relationship between the NPQ of Chl fluorescence and the WWC was elucidated by Schreiber's group. Addition of H_2O_2 to the illuminated chloroplasts stimulates the electron flux in PSII with the incident maximal yield of Chl fluorescence (F_m') decreased (Neubauer and Schreiber 1989). The decrease in F_m', the NPQ of Chl fluorescence, is suppressed by the inhibition of APX. The PET system activity to regenerate Asc induces ΔpH (Hormann et al. 1994). Furthermore, under anaerobic conditions, where O_2 photoreduction in the WWC is inhibited, the induction of NPQ of Chl fluorescence is completely suppressed (Schreiber et al. 1991). Schreiber's group termed the O_2-dependent electron transport system for the induction of NPQ of Chl fluorescence as the MAP pathway (Neubauer and Schreiber 1989). The MAP pathway is also known as the WWC. Subsequently, Sonoike's group identified a mutant of Arabidopsis that is deficient in WWC activity. The WWC-deficient mutant could not induce NPQ of Chl fluorescence (Higuchi et al. 2009). In addition, Hideg's group demonstrated the inactivation of APX in vivo at higher temperature and that heat-treated leaves did not show NPQ of Chl fluorescence (Hideg et al. 2008). These results also support Schreiber's hypothesis.

Schreiber proposed a mechanism for the induction of NPQ of Chl fluorescence, which differs from the theory that the xanthophyll cycle and PsbS protein contribute to the dissipation of the excess photon energy (Schreiber and Neubauer 1990). The acidification of the luminal side of thylakoid membranes lowers the efficiency of electron transport from H_2O to the oxidized reaction center P680. As a result, the increased oxidized P680 contributes to the dissipation of excess photon energy as heat, as observed in NPQ of Chl fluorescence. An examination of this hypothesis with regard to molecular mechanisms is still awaited.

The fourth physiological function of the WWC is the supply of ATP for assimilation of CO_2. Under anaerobic conditions, photosynthesis in algae and intact chloroplasts does not start (Heber and French 1968, Allen 1975, Egneus et al. 1975, Ziem-Hanck and Heber 1980, Laisk and Oja 1998). In addition, suppression of the WWC prolongs the induction period for CO_2 assimilation in higher plants (Makino et al. 2002). These results indicate that the activity of WWC is indispensable for driving photosynthesis.

For continuous CO_2 assimilation in C_3 plants, one of the substrates for Rubisco [ribulose-1,5-bisphosphate (RuBP)], has to be regenerated. Both PCR and PCO cycles regenerate RuBP using both NADPH and ATP. Furthermore, the requirement for ATP and NADPH in both cycles depends on the intercellular partial pressure of CO_2 (Ci). The ratio of consumption of ATP to that of NADPH in both cycles is calculated to be $(3+3.5\varphi)/(2+2\varphi)$ (von Caemmerer and Farquhar 1981). The value of φ is the ratio of the carboxylation rate of RuBP to its oxygenation rate by Rubisco. At the CO_2 compensation point φ is 2, and at higher Ci, it is zero, i.e. φ ranges from 0 to 2. Furthermore, the rate of ΔpH formation across thylakoid membranes in the light reaction should be at least equal to that of the ΔpH consumption rate in the dark reaction under steady-state CO_2 assimilation conditions (Miyake et al. 2005). For each turn of the Q cycle in the Cyt b_6/f complex of thylakoid

membranes, the uptake rate of protons to the luminal side against the electron flux in LEF should be 3. Furthermore, the number of protons required for the production of one molecule of ATP by ATP synthase is proposed to be 4.67 (Seelert et al. 2000, Scheuring et al. 2001). Under these conditions, the relationship between the production rate of luminal protons by LEF and the consumption rate of protons by both PCR and PCO cycles is shown in the following equation, where Jf is the electron flux in LEF and Jg is the electron flux in both PCR and PCO cycles.

$$3\,Jf = 2.385(3+3.5\,\varphi)/(2+2\varphi)\,Jg$$

At steady state, Jf should be equal to Jg. However, in the range of φ in vivo,

$$3\,Jf < 2.385(3+3.5\,\varphi)/(2+2\varphi)\,Jg$$

These results indicate that LEF alone cannot supply ATP to drive both PCR and PCO cycles (Miyake et al. 2005).

On the assumption that the WWC functions for the formation of extra ΔpH across thylakoid membranes, the required electron flux in the WWC (Jw), i.e. the electron sink ability of the WWC, against Jg is calculated as:

$$Jw/Jg = (2.385/3)\,(3+3.5\,\varphi)/(2+2\varphi) - 1$$

The dependence of Jw/Jg on φ is shown in **Fig. 2**. At higher Ci, WWC should express an electron flux of about 20% of Jg to drive CO_2 assimilation. On the other hand, at the CO_2 compensation point, the activity of the WWC is expected to increase to about 30% of the total electron flux in LEF. The fact that CO_2 assimilation in photosynthesis proceeds in the presence of O_2 reflects the requirement for ΔpH formation driven by the WWC, i.e., the MAP pathway (Makino et al. 2002).

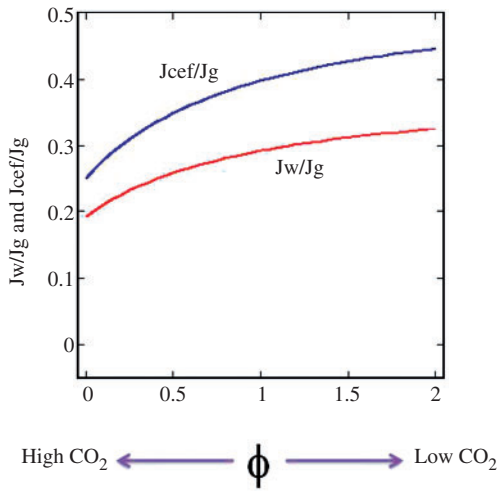

Fig. 2 Theoretical ratio of both Jw and Jcef to Jg, respectively, required for the net CO_2 assimilation at different φ values. To calculate both Jw/Jg and Jcef/Jg, 4.67 H^+/ATP and Q cycle activity were assumed (see text). The value of φ is the ratio of the rate of RuBP oxygenation to that of RuBP carboxylation by Rubisco. At high CO_2, where photorespiration does not function, φ is zero. An increase in φ means that the proportion of the photosynthetic linear electron flux diverted to the PCO cycle increases and both Jw/Jg and Jcef/Jg increase. At φ = 2, where the net CO_2 assimilation rate is zero, i.e. the CO_2 compensation point, Jw/Jg reaches 0.32 and Jcef/Jg reaches 0.45. Jg, the electron flux in both PCR and PCO cycles; Jw, that in the water–water cycle; Jcef, that in CEF.

Cyclic Electron Flow Around PSI

Molecular mechanism of CEF-PSI

In CEF-PSI (**Fig. 3**), the electrons photoproduced at PSI of thylakoid membranes return to the PET system through Fd or NADPH, where the electrons are donated to the PQ pool or Cyt b_6/f complex (Joliot and Joliot 2002, Breyton et al. 2006, Joliot and Joliot 2006). Initial research on CEF makes use of in vitro systems. Over 40 years ago, Arnon's group found that thylakoid membranes could synthesize ATP in the presence of Fd, ADP and Pi by illuminating only PSI, i.e. driving CEF (Arnon 1959, Tagawa et al. 1963). CEF in vitro is inhibited by the antibiotic antimycin A (Endo and Asada 2002). Furthermore, protonophores inhibits the synthesis of ATP. On the basis of homology of the inhibitory action of antimycin A on the Q cycle of the Cyt b/c_1 complex in mitochondria, it is proposed that the electrons from Fd in CEF flow to the PQ pool through the Cyt b_6/f complex, where low potential heme *b* is reduced by Fd and the reduced heme *b* donates electrons to PQ (Endo and Asada 2002, Heber 2002).

The model for the electron flow from Fd to the PQ pool in CEF is based on studies of Chl fluorescence in vitro (Endo and Asada 2002). In the presence of Fd, the addition of NAD(P)H to the thylakoid membranes of chloroplasts

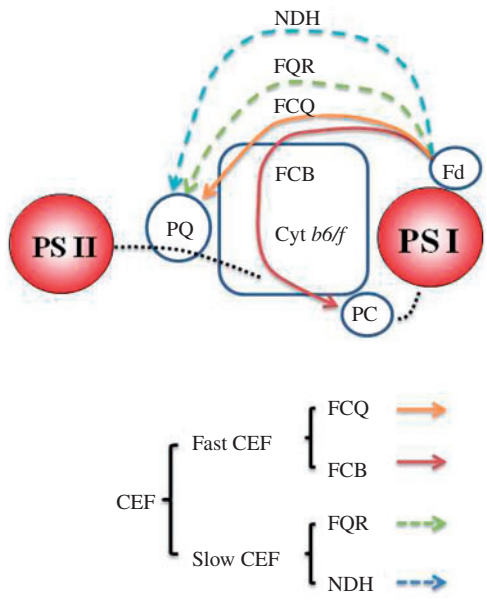

Fig. 3 Pathways in CEF. Solid arrows show fast CEF, dotted arrows show slow CEF. The yellow arrow is the electron flow pathway in FCQ (see text); red, FCB; green, FQR; blue, NDH.

increases the minimum yield of Chl fluorescence (F_o) (Hosler and Yocum 1985, Groom et al. 1993, Mano et al. 2005). The increase in F_o is suppressed by addition of antimycin A or by illumination with far-red light to excite PSI alone. These results indicate that NAD(P)H donates electrons to Fd through FNR, and the reduced Fd donates electrons to the PQ pool through the Cyt b_6/f complex. The electron flow from the reduced Fd to PQ was inhibited by antimycin A (Bendall and Manasse 1995). Furthermore, Asada's group showed that electrons photoproduced at PSI of thylakoid membranes flow to the PQ pool in intact chloroplasts and intact leaves (Asada et al. 1993, Mano et al. 1995, Field et al. 1998). After illumination with actinic light, the steady-state yield of Chl fluorescence (F_s) decreases to the minimum yield, and thereafter the minimum yield of Chl fluorescence transiently increases. These transient increases in the minimum yield reflect reduction of the PQ pool by NAD(P)H accumulated during actinic light illumination. These results obtained from in vitro experiments in thylakoid membranes or intact chloroplasts suggest the existence of CEF in leaves.

From in vitro research on CEF, intriguing results have been obtained (Hormann et al. 1994). CEF cannot function without electron input from PSII, i.e. CEF requires the concerted action of PSI and PSII. Far-red-dependent CEF activity is completely inhibited by DCMU. What does this mean? For CEF to function, Fd, PQ and the mediator catalyzing the electron flow from Fd to the PQ pool are required. Furthermore, the redox ratio of the PQ pool regulates CEF activity (**Fig. 4**; Allen 2003). The maximum activity of CEF is obtained at the half-reduced state of the PQ pool. On the other hand, under extreme conditions of the redox state, that is perfectly reduced and oxidized, CEF is not active, i.e. inhibition of CEF by DCMU is due to oxidation of the PQ pool (Hormann et al. 1994). Furthermore, these results indicate that the reduction rate of the PQ pool by Fd limits CEF activity at steady state in illuminated intact chloroplasts. The reasons for this limitation are: first, the ability of the mediator to catalyze the reduction of the PQ pool by Fd in CEF is lower than that of LEF to produce the reduced Fd or NADPH; and, secondly, electrons photoproduced at PSI flow to metabolic pathways other than CEF, resulting in a decrease in the electron flux to CEF and stimulation of oxidation of the PQ pool. The leak of electrons from CEF to other electron sinks suggests that CEF is not dominant in the PET system. To elucidate the kinetics of CEF and its physiological mechanisms and functions, the precise determination of CEF activity in vivo is essential.

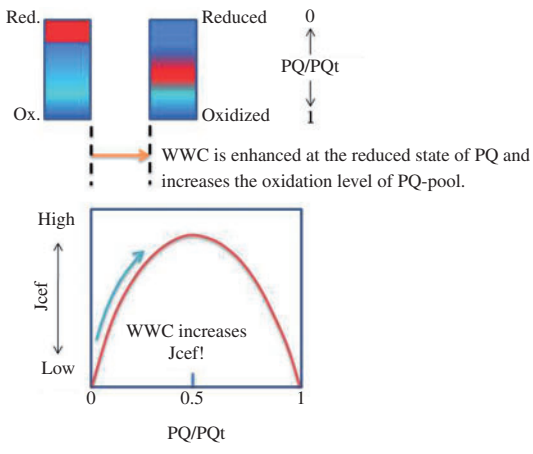

Fig. 4 Model of the expression of CEF activity: dependence of Jcef on the ratio (PQ/PQt) of oxidized PQ (PQ) to total PQ pool (PQt), and the regulation of Jcef by the WWC. Jcef is plotted against PQ/PQt according to the model of Allen (2002, 2003). In both the extremely oxidized state and the reduced state of PQ, Jcef is negligible. In the reduced state, where the light intensity is higher or the intercellular partial pressure of CO_2 is lower, the WWC is enhanced and functions as an electron sink, which contributes to the oxidation of PQ. The alleviation of the reduced state of PQ stimulates CEF.

I propose the following four mechanisms of CEF.

- CEF
  - Fast CEF
    - FCQ
    - FCB
  - Slow CEF
    - FQR
    - NDH (requiring Fd as electron donor)

With regard to the magnitude of electron flux in CEF, CEF is divided into two groups: (i) fast CEF, which has a large flux; and (ii) slow CEF, which has a small flux. Fast CEF is further divided into two pathways, FCQ and FCB. In the FCQ pathway, electrons photoproduced at PSI flow from Fd to the PQ pool through heme *c* in the Cyt b_6/f complex (Kurisu et al. 2003, Stroebel et al. 2003). In the FCB pathway, electrons flow from Fd to high potential heme *b* in the Cyt b_6/f complex through heme *c*, where CEF does not produce ΔpH across thylakoid membranes (Laisk et al. 2010). As described below, the electron flux in CEF in vivo reaches approximately 100–200 μmol $e^- m^{-2} s^{-1}$ (Miyake et al. 2005, Joliot and Joliot 2005, Laisk et al. 2010). This CEF activity cannot be accounted for by slow CEF. Furthermore, slow CEF is also divided into two groups [Fd-quinone oxidoreductase (FQR) and NAD(P)H dehydrogenase (NDH)]. In the FQR pathway, electrons photoproduced at PSI flow from Fd to the PQ pool through FQR (Heber and Walker 1992). FQR probably contain the related proteins, PGR5 and PGRL1 (Munekage et al. 2002, Shikanai 2007, Munekage et al. 2008, Okegawa et al. 2008, Dalcorso et al. 2010). Electron flow in the FQR pathway, as determined from in vitro experiments, is inhibited by antimycin A. However, antimycin A inhibits the LEF-dependent formation of NPQ of Chl fluorescence

(Oxborough and Horton 1987). These results confirmed the action of antimycin A on PSII. The multiple effects (inhibition of NPQ induction, disappearance of ΔpH across thylakoid membranes, inhibition of F_o increase) of antimycin A in the inhibition of photosynthesis need to be resolved. In the NDH pathway, electrons photoproduced at PSI flow from NAD(P)H to the PQ pool through FNR, Fd and NDH (Endo and Asada 2002). From in vitro research, Endo et al. (1998) proposed that Fd is required for the activity of the NDH pathway. Endo suggested that either NDH has the activity of FQR or NDH is FQR itself (Endo et al. 1998). Some subunits of plastid-localized NDH have homology with mitochondrial NDH (Endo and Asada 2002, Ishikawa et al. 2008, Shimizu et al. 2008, Ishida et al. 2009). However, the subunit required for the binding of NAD(P)H to the NDH complex, which is present in mitochondrial NDH, has not been found in the plastid-localized NDH.

In vivo activity of CEF

To elucidate the physiological function of CEF, the activity of CEF should be evaluated in vivo. Three methods for the determination of CEF activity have been proposed and all depend on the exact determination of the P700 turnover rate. The first was developed by Schreiber's group (Klughammer and Schreiber 1994). They defined the quantum yield of PSI [Φ(PSI)] to be the ratio of P700 charge separation under illuminated conditions [photo-redox active (pra-) P700] to total P700. To evaluate the pra-P700, they illuminated leaves with short, strong pulses. The half-rise time of the pulse was near 1 μs. The half-time for the reduction of $P700^+$ by the reduced PC ranged from 1 to 5 ms, i.e. the short pulse used by Schreiber's system enables us to determine the content of pra-P700 accurately. Schreiber's method differs from the conventional method (Harbinson et al. 1989, Harbinson et al. 1990) as in the conventional method the content of total P700 is evaluated by illumination with far-red light. Also, Φ(PSI) is defined to be equal to $[(P700)total-(P700^+)]/(P700)total$, where (P700)total is the total content of P700 and ($P700^+$) is that of the oxidized P700 under illumination conditions. The conventional method overestimates the value of Φ(PSI) because the content of pra-P700 is overevaluated under conditions where the electron transfer rate at the acceptor side of PSI limits the photo-redox cycle of P700. Under such conditions, the photoexcited P700, from the short pulse used in Schreiber' method, de-excites to the ground state via two roots. In the first, the excited P700 loses its energy as heat via charge recombination, which P700 does not contribute to the electron transfer step. In the second, the excited P700 gives electrons to the primary electron acceptor in PSI via charge separation, resulting in the conversion of itself to $P700^+$. The resulting $P700^+$ accepts electrons from the reduced PC, with the ground state P700 regenerated. Therefore, Φ(PSI) evaluated by Schreiber's method is lower than that by the conventional method, and its value is more precise. If Φ(PSI)/Φ(PSII) is >1, the extra Φ(PSI) would be due to the activity of CEF.

The second method to determine CEF activity was developed by Johnson's group (Johnson 2003, Nandha et al. 2007). They measured the reduction rate of $P700^+$ photoproduced at steady state. This method involves two problems (Baker et al. 2007): first is the difficulty in detection of $P700^+$ at lower light intensity; and, secondly the reduction kinetics of $P700^+$ are expressed by the multiple kinetic components, which is due to the sequential or parallel electron transfers from PC, the Cyt b_6/f complex or the reduced plastoquinol to $P700^+$.

The third method was developed by Joliot and Kramer's group. They estimated the activity of CEF from the rate of decay of the electrochromic shift which is induced by the formation of the membrane potential (Kramer et al. 2004, Joliot and Joliot 2006, Baker et al. 2007, Takizawa et al. 2007, Bailleul et al. 2010, Livingston et al. 2010). Details of this method are described elsewhere (Joliot and Joliot 2002, Cardol et al. 2008). The notable result obtained by this method is that CEF shows a higher activity in the induction phase of photosynthesis (Joliot and Joliot 2002). This is consistent with results obtained by Makino's group who used Schreiber's method (Makino et al. 2002).

The activity of CEF is expressed when the PET rate is limited by the electron sink activity. For example, the extra Φ(PSI) can be recognized in the dependence of the photosynthetic rate on light intensity (Clarke and Johnson 2001, Johnson 2005). With an increase in light intensity, net CO_2 assimilation rate increases, and its rate becomes saturated against the further increase in light intensity. On the other hand, the electron flux in PSI of thylakoid membranes continues to increase with the increase in light intensity, even after light intensity saturation of the net CO_2 assimilation rate (Johnson et al. 2005). That is, extra turnover of the redox cycle of P700, which is more than that required for LEF, occurs. The extra electron flux in PSI corresponds to the activity of CEF. In C_3 plants, the light intensity saturation of the photosynthesis rate under atmospheric CO_2/O_2 conditions implies that photosynthesis is limited by the carboxylation rate of RuBP by Rubisco. Furthermore, the CO_2 partial pressure saturation of the photosynthesis rate at higher light intensity implies that photosynthesis is limited by the regeneration rate of RuBP in the Calvin cycle (von Caemmerer and Farquhar 1981). Under these conditions, the regeneration rate of $NADP^+$ limits the electron flux in LEF.

Furthermore, due to the dependence of the photosynthesis rate on CO_2 partial pressure, the assimilation rate of CO_2 decreases with a decrease in the Ci (von Caemmerer 2000). With the decrease in Ci, the value of Φ(PSI) relative to Φ(PSII) [Φ(PSI)/Φ(PSII)] increases. This results in enhancement of CEF under natural conditions. For example, under drought conditions where stomata of the leaves close, Φ(PSI)/Φ(PSII) increases (Golding and Johnson 2003, Rumeau et al. 2007). Furthermore, on exposure to lower temperatures, CEF activity is enhanced (Hirotsu et al. 2005, Johnson et al. 2005). At lower temperatures, Rubisco activities of both RuBP carboxylase and oxygenase decrease, with the regeneration rate of $NADP^+$ suppressed, and its suppression limits the electron flux in LEF, i.e. the suppression of photosynthesis would induce CEF activity.

Next, I consider the expression of CEF activity using the model proposed by Allen (2003) (**Fig. 4**). The limitation of the

electron flux in LEF by the regeneration rate of $NADPH^+$ induces accumulation of electrons at the reducing side of PSI of thylakoid membranes, i.e. the electron donor in CEF, the reduced form of Fd, accumulates. As a result, the redox ratio of the PQ pool shifts to the reduced state. As shown in **Fig. 4**, an increase in the reduced state of the PQ pool enhances the CEF activity. In the present model, with extreme reduction of the PET system, the activity of CEF is expected to be suppressed, which probably reflects that the reduced PQ in PET cannot accept any more electrons from the reduced form of Fd in CEF. Thus these results, deduced from the model, suggest that at the extreme suppressed state of photosynthesis CEF cannot function.

Here, I propose a model for the regulation of CEF activity by the WWC (**Fig. 4**). The activity of the WWC increases at the suppressed electron flux in LEF, and contributes to the oxidation of the PQ pool (Miyake and Yokota 2000). Furthermore, the activity of the WWC competes with that of CEF (Hormann et al. 1994, Makino et al. 2002). These results suggest the allocation of electrons at PSI to both the WWC and CEF. The WWC oxidizes the PQ pool for the turnover of CEF and its requirement for PQ pool oxidization increases under suppressed conditions of photosynthesis, i.e. unless the WWC functions, CEF cannot be active.

Physiological functions of CEF

It has been proposed that there are two physiological functions of CEF in thylakoid membranes (Heber and Walker 1992, Heber 2002, Breyton et al. 2006, Joliot and Joliot 2006, Baker et al. 2007, Eberhard et al. 2008, Kohzuma et al. 2008, Livingston et al. 2010). First is the supply of ATP required for regeneration of RuBP to support net CO_2 assimilation in photosynthesis. Second is the induction of NPQ of Chl fluorescence under conditions where the regeneration rate of $NADP^+$ limits the electron flux in LEF. Later, Laisk proposed a third physiological function of CEF, i.e. CEF suppresses the production of O_2^- in the WWC (Laisk et al. 2010).

In CEF, the PQ pool is reduced by Fd which is photoreduced by PSI. The reduced PQ pool is oxidized by the Cyt b_6/f complex, where ΔpH across thylakoid membranes through the Q cycle is induced. This ΔpH drives ATP synthase and produces the ATP required for the net CO_2 assimilation. The required level of CEF for photosynthesis is shown in **Fig. 2** (Miyake et al. 2005).

On the assumption that CEF contributes to the induction of ΔpH across thylakoid membranes, the required activity of CEF (Jcef) for net CO_2 assimilation is estimated as follows.

$$Jcef/Jg = (4.67/4)\,(3 + 3.5\,\varphi)/(2+2\varphi) - 1.5$$

Dependence of Jcef/Jg on φ is shown in **Fig. 2**. At the higher partial pressure of CO_2, photosynthesis requires about 25% of CEF against the electron flux in total LEF, Jg. On the other hand, at the lower partial pressure of CO_2, for example the CO_2 compensation point, about 45% of CEF against Jg is required for the turnover of both PCR and PCO cycles, which is much higher than that at higher Ci. In tobacco leaves, Jcef/Jg was about 40% at $\varphi=0$ and about 60% at the CO_2 compensation point at higher light intensity (Miyake et al. 2005). However, at lower light intensity, Jcef/Jg was about 20% at $\varphi=0$ and about 20% at $\varphi=2$. Thus its activity did not change in response to changes in Ci (Miyake et al. 2005). That is, it is clear that the measured activity of CEF cannot satisfy all the required ATP to drive net CO_2 assimilation. I hypothesize that the WWC helps CEF to supply ATP in net CO_2 assimilation, i.e. the WWC is indispensable not only for CEF to function at the extreme redox state of the PQ pool, but also for both PCR and PCO cycles to turn over in coordination with CEF. However, if the stoichiometry of protons required for the production of ATP in chloroplast ATP synthase, H^+/ATP, is 4, the WWC alone can satisfy the requirement.

Cornic et al. (2000) demonstrated the contribution of CEF to the induction of NPQ of Chl fluorescence. Illumination of leaves or intact chloroplasts by far-red light to excite only PSI of thylakoid membranes induces NPQ with the enhancement of electrochromic shift, which shows the induction of ΔpH across thylakoid membranes. In addition, CEF activity responds to the environment (Rumeau et al. 2007, Jia et al. 2008, Kohzuma et al. 2008). Tobacco plants grown under higher intensity light showed a larger value of NPQ of Chl fluorescence with enhanced activity of CEF, compared with plants grown under the lower light intensity. These results suggest that the enhancement of CEF activity is indispensable for the induction of NPQ. Furthermore, we succeeded in boosting the activity of CEF, the first time this had been achieved, by overexpressing the redox protein Fd in chloroplasts of tobacco leaves (Yamamoto et al. 2006). The enhancement of Fd content in chloroplasts increased not only fast CEF activity, but also slow CEF, as observed in the post-illumination increase of minimum Chl fluorescence yield. We also succeeded in the enhancement of NPQ of Chl fluorescence. These results are direct proof that CEF contributes to the induction of NPQ.

However, the enhancement of CEF activity failed to magnify the net CO_2 fixation (Yamamoto et al. 2006). I therefore hypothesize that wild plants have sufficient CEF activity for the turnover of both PCR and PCO cycles.

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