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The water–water cycle as alternative photon and electron sinks

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The water–water cycle in chloroplasts is the photoreduction of dioxygen to water in photosystem I (PS I) by the electrons generated in photosystem II (PS II) from water. In the water–water cycle, the rate of photoreduction of dioxygen in PS I is several orders of magnitude lower than those of the disproportionation of superoxide catalysed by superoxide dismutase, the reduction of hydrogen peroxide to water catalysed by ascorbate peroxidase, and the reduction of the resulting oxidized forms of ascorbate by reduced ferredoxin or catalysed by either dehydroascorbate reductase or monodehydroascorbate reductase. The water–water cycle therefore effectively shortens the lifetimes of photoproduced superoxide and hydrogen peroxide to suppress the production of hydroxyl radicals, their interactions with the target molecules in chloroplasts, and resulting photoinhibition. When leaves are exposed to photon intensities of sunlight in excess of that required to support the fixation of CO_2, the intersystem electron carriers are over-reduced, resulting in photoinhibition. Under such conditions, the water–water cycle not only scavenges active oxygens, but also safely dissipates excess photon energy and electrons, in addition to downregulation of PS II and photorespiration. The dual functions of the water–water cycle for protection from photoinhibition under photon excess stress are discussed, along with its functional evolution.

Keywords: active oxygens; alternative electron flux; dissipation, excess photon energy; excess photon stress; photoinhibition; water–water cycle

1. INTRODUCTION

Photosynthetic organisms are always exposed to varying environmental factors. Sunlight intensity especially varies from moment to moment, and plants need to respond quickly to such changes to escape from photoinhibition. Plants are able to adapt by acclimation in the long term (i.e. over hours or days) to light environments within a genetically defined range, but plants must also respond to rapid changes of sunlight intensity within seconds or minutes. In principle, plants suffer photoinhibition under conditions where exposed photon intensity (photosynthetically active photon flux density, PPFD) is in excess of that required to support observed rates of CO_2 assimilation (A), i.e. photon use capacity of plants. Under conditions where PPFD is less than or equal to that required to support observed rates of A, most of the absorbed photon energy is used for CO_2 assimilation. However, when PPFD is in excess of that required to support observed rates of A, the intersystem electron carriers are over-reduced and excess photon energy generates reactive species of oxygen to bring about photoinhibition, if their generation is in excess of their scavenging capacities of plants. *In vivo*, the conditions where PPFD is in excess of that required to support observed rates of A are not unusual under direct sunlight, and even shade plants are transiently exposed to photon excess conditions by sunfleck. Photon using capacity, A, is not constant for each plant, and varies depending on its developmental stage, age, tissues and cells. Even within a leaf, parenchyma and spongy cells show different responses to light. Furthermore, it is largely affected by other environmental factors than sunlight—such as temperature, drought, mineral nutrition (Cakmak 2000), atmospheric concentration of CO_2 in the canopy, CO_2 transport to chloroplasts, sink stress and pollutants—and these factors are also variable at every moment. Therefore, a balance between PPFD and A is also variable from moment to moment.

For adaptation within seconds and minutes to photon excess stress to avoid photoinhibition, plants have prompt responsive mechanisms that can safely dissipate excess photon energy or excess electrons generated in thylakoids to suppress the production of reactive species of oxygen. A rapid response to dissipate excess photons is downregulation of the quantum yield of PS II initiated by the proton gradient across the thylakoid membranes and by its associated de-epoxidation of violaxanthin (Horton *et al.*, this issue). Photorespiration also dissipates excess photons and electrons by consumption of NADPH and ATP via the photorespiratory pathway, while at the same time supplying the electron acceptor, CO_2, to chloroplasts (Osmond 1981; Kozaki & Takeba 1996).

In this paper, the molecular mechanism of the water–water cycle, which photoreduces dioxygen to water without release of superoxide and hydrogen peroxide from their generation sites, is reviewed. The water–water cycle is indispensable for suppressing the photoinhibition

by reduced, reactive species of oxygen generated in chloroplasts. In addition, the water–water cycle participates in the dissipation of excess photons and electrons as an alternative electron flux. The dual functions of the water–water cycle for protection from photoinhibition are discussed.

2. MOLECULAR MECHANISM AND CHARACTERISTICS OF THE WATER–WATER CYCLE IN CHLOROPLASTS

The photoreduction of O_2 to hydrogen peroxide in thylakoids was discovered half a century ago by Mehler (1951), which was the first finding that chloproplasts not only oxidize water to evolve O_2 but also reduce O_2. However, for over two decades the photoreduction of O_2 was considered to represent a minor electron flux and an unavoidable reaction in chloroplasts. ATP formation accompanying the linear electron flow to O_2—pseudocyclic photophosphorylation—was supposed to be its sole physiological function. In the 1970s, the superoxide radical was identified as the primary product of the photoreduction of O_2 by several methods (for a review, see Asada & Takahashi 1987), and occurrence of its scavenging enzyme Cu,Zn-superoxide dismutase (Cu,Zn-SOD) in chloroplasts was shown (Asada *et al.* 1973). Following this, inactivation of several Calvin cycle enzymes and inhibition of CO_2 fixation by hydrogen peroxide was demonstrated (Kaiser 1976), the first identification of the target molecules of active oxygens in chloroplasts. At the same time, occurrence of glutathione reductase in chloroplasts was found, and ascorbate was proposed as a scavenger of reduced species of oxygen in chloroplasts (Foyer & Halliwell 1976). Localization of dehydroascorbate (DHA) reductase in chloroplasts and its participation in scavenging of hydrogen peroxide were then confirmed (Nakano & Asada 1981; Jablonski & Anderson 1981). In addition to DHA reductase, the participation of monodehydroascorbate (MDA) reductase (Marre & Arrigoni 1958) in the regeneration of ascorbate of chloroplasts was also established (Hossain *et al.* 1984).

(a) *Peroxidase in chloroplasts*

In plant cells a haem catalase exists in peroxisomes but not in chloroplasts. For suppression of the inhibition of CO_2 fixation, the hydrogen peroxide produced via the SOD-catalysed disproportionation of superoxide should be immediately removed by either catalase or peroxidase. Photosystem II (PS II) membranes show a catalatic activity (disproportionation of hydrogen peroxide to O_2 and water), when the membranes are excited to the S_2 state. The oxidizing equivalents generated in PS II membranes compete between water and hydrogen peroxide for the oxygen evolution from them (Mano *et al.* 1987, 1993). This catalatic activity, however, requires a high concentration of hydrogen peroxide to suppress the oxygen evolution from water (0.4 mM for half inhibition). On the other hand, a half inhibition of CO_2 fixation by hydrogen peroxide occurs at only 10 μM (Kaiser 1976), and so the functional participation of this catalatic activity in the scavenging of hydrogen peroxide is unlikely.

The participation of peroxidase in the scavenging of hydrogen peroxide was confirmed by photoreduction of $^{18}O_2$ at the same rate of as that of $^{16}O_2$ evolution from water and by the evolution of expected stoichiometric evolution of $^{16}O_2$ from water on addition of $H_2{}^{18}O_2$ in intact chloroplasts (Asada & Badger 1984). Thus, chloroplasts can reduce O_2 to water via superoxide and hydrogen peroxide in PS I by the electrons derived from water in PS II, with little diffusion of superoxide and hydrogen peroxide from their generation sites.

Since the regenerating enzymes of ascorbate occur in chloroplasts, ascorbate is the most likely electron donor in the peroxidase reaction for scavenging hydrogen peroxide. Ascorbate peroxidase (APX) was found by Kelly & Latzko (1979) in a soluble form and by Groden & Beck (1979) in a thylakoid-bound form in plants. The peroxidase for scavenging hydrogen peroxide in chloroplasts has been identified to be the enzyme using ascorbate as the specific electron donor (Nakano & Asada 1981). Classical plant peroxidase, guaiacol peroxidase (as represented by horseradish peroxidase) is not present in chloroplasts, but APX is a new family of peroxidase which scavenges hydrogen peroxide, including cytochrome *c* peroxidase from yeast and catalase peroxidase from prokaryotes (Mutsuda *et al.* 1996). APX is distinguished from guaiacol peroxidase in physiological function, amino-acid sequence and specificity of electron donor (Asada 1997).

(b) *Whole sequences of the water–water cycle*

The molecular mechanism of the water–water cycle and microcompartmentation of the participating enzymes in chloroplasts are illustrated in figure 1. For further details of the cycle and the participating enzymes in it, see a recent review by Asada (1999).

The water–water cycle comprises the following reaction sequences:

$$2H_2O \rightarrow 4[e^-] + O_2 + 4H^+ \quad (1)$$

(oxidation of water in PS II),

$$2[e^-] + 2O_2 \rightarrow 2O_2^- \quad (2)$$

(the photoreduction of dioxygen to superoxide in PS I by the electrons derived from PS II),

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

(the disproportionation of superoxide to hydrogen peroxide and dioxygen catalysed by SOD),

$$H_2O_2 + 2 \text{ ascorbate} \rightarrow 2H_2O + 2 \text{ monodehydroascorbate} \quad (4)$$

(the reduction of hydrogen peroxide to water by ascorbate catalysed by APX), and

$$2[e^-] + 2H^+ + \text{oxidized ascorbates} \rightarrow \text{ascorbate} \quad (5)$$

(the regeneration of ascorbate from oxidized forms of ascorbate, monodehydroascorbate and/or dehydroascorbate, by reduced ferredoxin and/or NAD(P)H photogenerated in PS I, which are reduced by the electrons from PS II).

The sum of the reactions (1) to (5) is

$$2H_2O + O_2^* \rightarrow O_2 + 2H_2O^*. \quad (6)$$

In the water–water cycle, half of the four electrons generated in PS II from two molecules of water are used

Table 1. *Local concentrations (5 nm layer on thylakoid membranes) and reaction rate constants of the scavenging enzymes, and simulated half-lives of O_2^-, H_2O_2 and monodehydroascorbate radical (MDA) in the thylakoidal scavenging system of spinach chloroplasts (Asada 1999)*

reaction	local concentration of enzyme (mM)	reaction rate constant: second order ($M^{-1}s^{-1}$)	reaction rate constant: pseudo first order (s^{-1})	half-life of substrate (s)
$SOD + O_2^-$	1	2.4×10^8	2.4×10^5	3×10^{-6}
$APX + H_2O_2$	1	10^7	10^4	7×10^{-5}
Fd + MDA	3	10^7	3×10^4	2×10^{-5}

Table 2. *Effect of thylakoid addition on the bicarbonate-dependent oxygen evolution by intact spinach chloroplasts (Asada 1992)*

(The photogeneration rate of hydrogen peroxide from superoxide by added thylakoids was 9 nM s^{-1} in the medium (1 ml), but that by intact chloroplasts was expected to be 0.12 mM s^{-1} within chloroplasts as estimated by the uptake rate of $^{18}O_2$ and a chloroplast volume of 25 µl mg Chl^{-1}. Thus, the hydrogen peroxide generated by the thylakoids within chloroplasts is so effectively scavenged through the water–water cycle that it does not inhibit CO_2 fixation (bicarbonate-dependent O_2 evolution), as observed no effect of catalase on it. On the contrary, the hydrogen peroxide photogenerated by thylakoids in the medium inhibits CO_2 fixation of the intact chloroplasts severely, even at a low concentration.)

reaction mixture	bicarbonate-dependent O_2 evolution (µmol mg $Chl^{-1}h^{-1}$)
intact chloroplasts (37 µg chlorophyll ml^{-1})	97
intact chloroplasts + catalase (1000 units ml^{-1})	97
intact chloroplasts + thylakoids (7 µg chlorophyll ml^{-1})	12
intact chloroplasts + thylakoids + catalase	76
thylakoids	−7

in the univalent reduction of two molecules of O_2 (in reaction (2)), and the remaining half in the regeneration of ascorbate from oxidized ascorbates (in reaction (5)). Thus, the water–water cycle does not give a net exchange of oxygen, and can be observed only by using $^{18}O_2$. This equal donation of the electrons to the two reactions is possible only when the rates of reactions (3), (4) and (5) are faster than that of reaction (2). If the rates of reactions (3), (4) and (5) are slower than that of photoreduction of O_2, the lifetime of superoxide would be prolonged and hydrogen peroxide would accumulate in chloroplasts. This does not occur in intact chloroplasts because when $^{18}O_2$ is photoreduced, $H_2{}^{18}O_2$ does not accumulate—its accumulation occurs only either when chloroplasts are ruptured and microcompartmentation of the scavenging enzymes is disrupted or when the inhibitor of APX and Cu,Zn-SOD cyanide is added (Asada & Badger 1984).

(c) *Microcompartmentation in chloroplasts of the participating enzymes in the water–water cycle*

Rapid disproportionation of superoxide, reduction of hydrogen peroxide and reduction of oxidized forms of ascorbate are guaranteed by the high reaction rates of the respective enzymes (table 1) and by their microcompartmentation in the vicinity of the generation site of superoxide PS I complex (figure 1). SOD catalyses the disproportionation of superoxide at 2×10^9 $M^{-1}s^{-1}$, which is the highest rate constant among not only the participating enzymes in the water–water cycle but also all enzymes known so far. The SOD reaction is diffusion controlled, but its actual reaction rate constant would be lowered one order of magnitude in the chloroplast stroma where the relative viscosity to that of water is 69 due to the high protein content of 40%. The second-order reaction rate constant of SOD in the stroma is estimated to decrease to around 0.12 of that in water, i.e. 2.4×10^8 $M^{-1}s^{-1}$ (Ogawa *et al.* 1995).

Little diffusion of superoxide from its generation site is guaranteed also by the attachment of SOD on the PS I-rich, stromal thylakoids (Ogawa *et al.* 1995). Attachment of over 70% of soluble SOD in chloroplasts to the stromal thylakoid membranes would be facilitated by ionic interactions of SOD molecules with the membranes. In such a way, the diffusion of the superoxide generated on the surface of the thylakoids is very limited, which is indispensable for avoiding inactivation of the hydrogen peroxide-sensitive enzymes in the stroma. Antisense-repressed transformant tobacco of chloroplastic Cu,Zn-SOD is susceptible to bright light as compared with the wild-type plants, and PS I is inactivated rather than PS II. Similar high sensitivity to strong light has also been observed in the transformant in which mitochondorial Mn-SOD is over-expressed in the chloroplasts but the chloroplastic Cu,Zn-SOD is repressed (Ogawa *et al.* 1997). These observations strongly support the requirement for the attachment of Cu,Zn-SOD to the thylakoid membranes in the vicinity of the PS I complex for effective scavenging of superoxide and protection from photoinhibition.

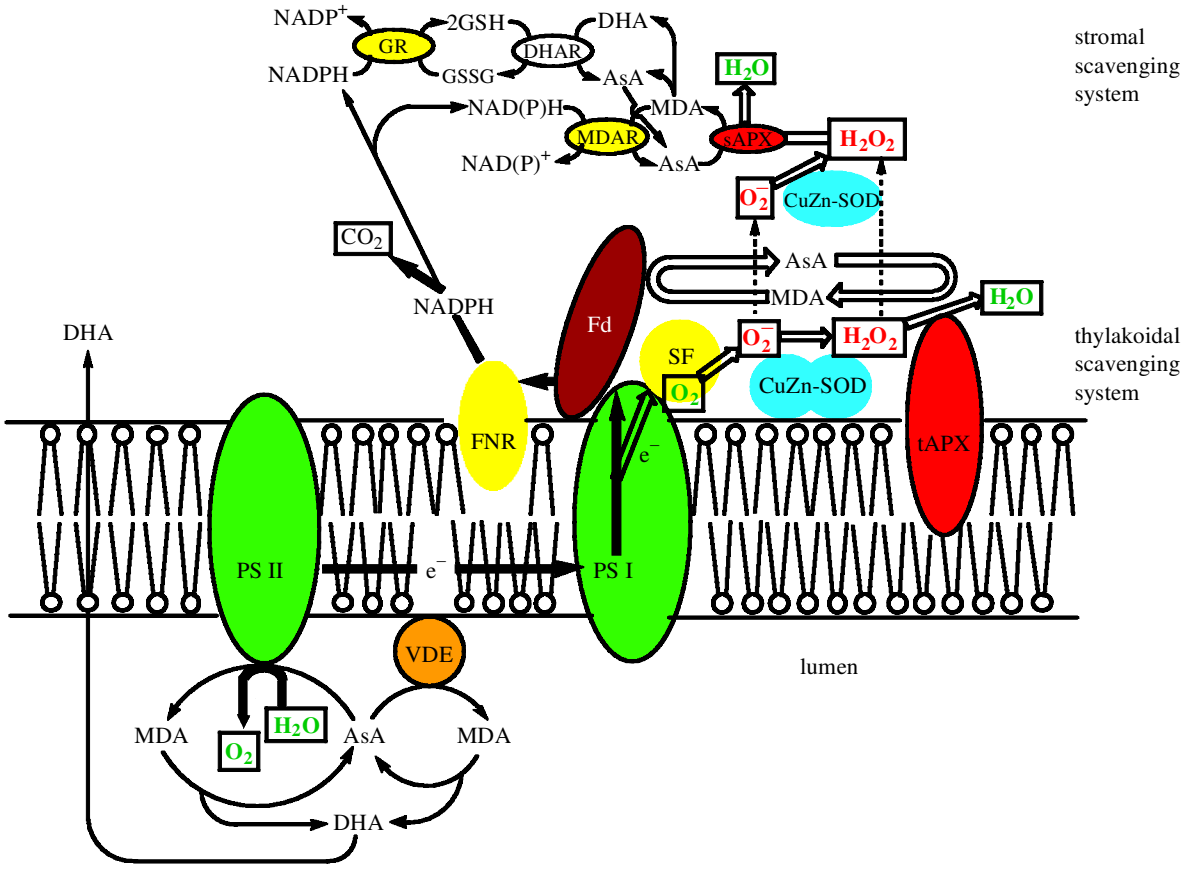

Figure 1. Water–water cycle in chloroplast, showing photoreduction of dioxygen to generate superoxide, disproportionation of superoxide to hydrogen peroxide, reduction of hydrogen peroxide to water and the associated reduction of oxidized ascorbates to ascorbate, and microcompartmentation of the participating enzymes. APX, ascorbate peroxidase; AsA, ascorbate; Cu,Zn-SOD, copper-zinc superoxide dismutase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; Fd, ferredoxin; FR, Fd-NADP$^+$ reductase; GR, glutathione reductase; MDA, monodehydroascorbate radical; MDAR, MDA reductase; sAPX, stromal APX; SF, stromal factor for enhanced photoreduction of dioxygen; tAPX, thylakoid-bound APX; VDE, violaxanthin de-epoxidase.

The hydrogen peroxide produced from superoxide via the SOD-catalysed reaction is also rapidly reduced to water by ascorbate, catalysed by the APX. Chloroplasts contain ascorbate at 10–30 mM which is high enough to saturate the APX reaction at $10^7\,M^{-1}s^{-1}$. In chloroplasts, two isoforms of APX occur: thylakoid bound and stromal forms (Miyake *et al.* 1993); the bound form has an additional hydrophobic domain in the C-terminus for binding to the membranes (Ishikawa *et al.* 1997). As shown in figure 1, the thylakoid-bound APX is the key enzyme for reduction of hydrogen peroxide and is microcompartmented with the attached Cu,Zn-SOD on the PS I complex of thylakoids (thylakoidal scavenging system). However, the stromal APX is a component of the stromal scavenging system. The thylakoidal scavenging system contains nearly one molecule each of SOD and the bound APX for one molecule of P_{700}, and the local concentration of SOD and APX in 5 nm layer on the thylakoid membranes are both *ca.* 1 mM and that of ferredoxin is *ca.* 3 mM.

Table 1 summarizes a simulation of the rates of the disproportionation of superoxide, the reduction of hydrogen peroxide to water and the reduction of monodehydroascorbate radical to ascorbate in the thylakoidal scavenging system, considering the viscosity of the stroma for the SOD reaction. Using these values, the pseudo first-order reaction rate constants and the half-lives are estimated. SOD of the thylakoidal scavenging system lowers the steady-state concentration of superoxide below 30 nM on 5 nm layer (the diameter of Cu,Zn-SOD) on the thylakoid membranes when the production rate of superoxide is 25 O_2^- $P_{700}{}^{-1}\,s^{-1}$ (125 μmol O_2^- mg $Chl^{-1}\,h^{-1}$; see table 3). If the SOD is uniformly distributed in the stroma, the concentration of superoxide on the 5 nm layer of thylakoid membranes is increased to over 150 nM and diffuses out from this layer where the target enzymes of the Calvin cycle are localized. In addition to the stoichiometry of $^{18}O_2$ experiments, other evidence for the short lifetimes of the intermediates of the water–water cycle is an observation that electron paramagnetic resonance signal of the monodehydroascorbate radicals (MDA) is not detectable in healthy leaves on illumination, indicating its rapid reduction to ascorbate in chloroplasts, although MDA appears under environmental stress (Heber *et al.* 1996) including ultraviolet (Hideg *et al.* 1997).

It should be noted that the simulated half-lives of superoxide, hydrogen peroxide, and monodehydroascorbate radicals, i.e. 10^{-5} to 10^{-6} s, are shorter than that of the maximal rate of the linear electron transport, i.e. 5×10^{-3} s. Thus, the limiting step of the water–water cycle is the reducing step of O_2, but not the disproportionation of superoxide, nor reduction of hydrogen peroxide

Table 3. *Comparison of the rates of the photoreduction of dioxygen to superoxide, in thylakoids and chloroplasts in leaf tissues; their rates, apparent K_m-values for oxygen and saturated intensity of light (Asada 1999)*

(The values for the chloroplasts are those from table 4.)

	production rate of O_2^- (O_2^-; $P_{700}{}^{-1}s^{-1}$)	K_m for O_2 (μM)	K_m for O_2 (kPa)	saturated light intensity (photon μmol $m^{-2}s^{-1}$)
thylakoids	4	2–10	0.2–0.8	10
chloroplasts	12–100	60–120	65–10	< 300

nor regeneration of ascorbate. This allows electrons derived from water in PS II to be divided into one-half for the reduction of oxygen and the remaining half for the regeneration of ascorbate.

The thylakoidal and stromal scavenging systems have the same stoichiometry for the reduction of O_2 to water in respect of the reducing equivalents, although the respective reducing systems of oxidized ascorbates are different from each other (figure 1). DHA reductase seems to play a role in the ascorbate generation from the DHA produced by the spontaneous disproportionation of MDA in the stromal system. DHA is generated from MDA also in the lumen by the violaxanthin de-epoxidase (VDE) of the xanthophyll cycle and by the donation of electrons to PS II from ascorbate in place of water (Mano *et al.* 1997) (figure 1). The DHA reductase-depleted tropical fig shows photobleaching of chlorophyll under direct sunlight (Yamasaki *et al.* 1999), indicating a requirement of DHA reductase for protection from photoinhibition.

It is not known whether the stromal system is uniformly distributed in the stroma or microcompartmented around the thylakoid membranes. However, as shown in table 2, when hydrogen peroxide diffuses to intact chloroplasts through the envelope from the suspending medium, the CO_2 fixation is severely inhibited even at low concentrations, as compared with that generated by the thylakoids in intact chloroplasts. This observation indicates the absence of the stromal scavenging system in the vicinity of the chloroplast envelope, and it is likely that the stromal system also microcompartments around the thylakoid membranes. It also remains to be established whether the stromal system is just for the 'second defence' of the thylakoidal system or for the scavenging of the reduced species of oxygen produced in the stroma. Another possible function of the stromal system is the scavenging of the reduced species of oxygen if produced in the dark (night) via the chlororespiratory process (Buchel & Garab 1996).

(d) *Characteristics of the water–water cycle*

These can be summarized as follows.

(i) The rate of the cycle is regulated by the photoreduction of O_2, but not by the disproportionation of superoxide, nor the reduction of hydrogen peroxide nor the reduction of oxidized forms of ascorbate. Therefore, the regeneration of ascorbate is always associated with the photoreduction of dioxygen, and the water–water cycle allows twofold linear electron flux.

(ii) The half-lives of superoxide and hydrogen peroxide are so short that they do not diffuse from their generation site, and active oxygens have little chance for interaction with the target molecules in chloroplast stroma and the PS I complex.

(iii) ΔpH and ATP are generated but no NADPH is generated, which has been referred to as 'peudocyclic electron flow' or 'pseudocyclic photophosphorylation'.

3. STROMAL FACTOR-MEDIATED PHOTOREDUCTION OF DIOXYGEN

Photoreduction of dioxygen in washed thylakoids is characterized by its low rate, its low apparent K_m for O_2 and its low intensity of saturating light (table 3). The photoproduction rate of superoxide by thylakoids is *ca.* 20 μmol mg chlorophyll^{-1} h^{-1}, corresponding to 4 O_2^- $P_{700}{}^{-1}s^{-1}$, and then accounts for the linear electron flux of only 8 $e^{-1}P_{700}{}^{-1}s^{-1}$ through the water–water cycle. However, the actual observed rates through the water–water cycle in leaf tissues and algal cells are higher than that by thylakoids only (table 4). The most remarkable case is *Scenedesmus* cells. The illuminated cells showed the same rate of $^{16}O_2$ evolution from water in both the presence and absence of CO_2, but, in the absence of CO_2, the rate of $^{18}O_2$ uptake is increased to the same rate as that of $^{16}O_2$ evolution (Radmer *et al.* 1978). This stoichiometry is the same as that observed in intact spinach chloroplasts when the water–water cycle operates (Asada & Badger 1984). The observed K_m for O_2 in the algal cells was 7 kPa, indicating that the $^{18}O_2$ uptake is not due to the oxygenase reaction of ribulose biphosphate carboxylase/oxygenase (rubisco). The same was observed also just after the turning on of light prior to the photoactivation of the Calvin cycle enzymes (Radmer & Kok 1976). Thus, at least, in these algal cells, the linear electron flux for the water–water cycle under the conditions of either CO_2 depletion or little CO_2 fixation is the same as that for CO_2 fixation. These electron fluxes via the water–water cycle cannot be accounted for by the photoreduction of dioxygen by thylakoids only—a stromal factor should participate in the enhancement of the photoreduction rate of dioxygen to meet the observed rates.

Auto-oxidation of the primary electron acceptor of PS I, [4Fe–4S] centre X or A/B, is the most likely electron donor for the photoproduction of superoxide in washed thylakoids. In chloroplasts what stromal components participate in the enhanced photoreduction of dioxygen? Auto-oxidation of reduced ferredoxin is an

Table 4. *Electron flux through the water–water cycle in algal cells and plant leaf tissues as per cent of the total linear electron flux and apparent K_m-values (O_2) of the photoreduction of dioxygen under various environmental conditions*

(For the data using $^{18}O_2$, see also Badger *et al.* (this issue). The values in square brackets represent the linear electron flux on the basis of $P_{700}{}^{-1}\,s^{-1}$. A dash indicates when the K_m for O_2 and the electron flux for the water–water cycle are not deduced or the experiments were done under non-specified environmental stress.)

species	environmental condition	K_m (O_2) (kPa)	linear electron flux through water–water cycle (% of linear electron flux, $e^-\ P_{700}{}^{-1}s^{-1}$)	reference
algae				
Synechococcus	saturating light	—	32	Mir *et al.* (1995)
Scenedesmus	CO_2 depletion	7	100 [80]	Radmer *et al.* (1978)
Chlamydomonas	—	—	?20	Sültemeyer *et al.* (1993)
Chondrus crispsis	—	4–16	—	Brechignac & Andre (1985)
Scenedesmus	before start CO_2 fixation after turning on of light	—	100	Radmer & Kok (1976)
C_4 plants				
maize (mesophyll)	—	—	—	Ivanov & Edwards (1997)
maize	—	5–10[a]	—	Maroco *et al.* (2000)
maize	chilling	—	—	Fryer *et al.* (1998)
sorghum	low CO_2	—	30	Laisk & Edwards (1998)
amaranthus	low CO_2	—	70	Laisk & Edwards (1998)
C_3 plants				
wheat	drought	—	29 [50]	Biehler & Fock (1996)
Indian mustard	low CO_2	7	30 [50]	Canvin *et al.* (1980) Osmond & Grace (1995)
sunflower	—	10	25–30	Laisk & Loreto (1996)
tomato	drought	—	—	Biehler *et al.* (1997)
tobacco	low rubisco	—	0	Ruuska *et al.*(2000)
pea	—	—	—	Park *et al.* (1996)
soybean	—	7–8	—	Behrens *et al.* (1982)
watermelon	low CO_2, bright light	8	20 [24]	Miyake & Yokota (2000)
Festucaprutensis	senescence	—	—	Kingston-Smith *et al.* (1997)
grape	chilling, drought	—	—	Flexas *et al.* (1999)
mangrove	salt stress (field)	—	30	Cheeseman *et al.* (1997)
tropical trees	—	—	10–20	Lovelock & Winter (1996)

[a] A range of optimal concentrations for maximal CO_2 fixation.

additional source of superoxide, and its concentration requirement of oxygen for the generation of superoxide is higher than that of thylakoids (Furbank & Badger 1983). However, because of its slow auto-oxidation rate ($0.08\,s^{-1}$ in 21 kPa O_2) and its rapid reaction rate with MDA for regeneration of ascorbate ($10^7\,M^{-1}s^{-1}$; Miyake & Asada 1994), this is not the major source of enhanced photoproduction of superoxide.

Recently, Miyake *et al.* (1998) found that flavodehydrogenases in chloroplasts enhanced the photoreduction of dioxygen producing superoxide by thylakoids: ferredoxin-$NADP^+$ reductase; glutathione reductase; and MDA reductase (Kobayashi *et al.* 1995; Sano *et al.* 1995). The NAD(P)H-reduced flavodehydrogenases are only slowly auto-oxidized, but the photoreduced enzymes by PS I of thylakoids are rapidly auto-oxidized to produce superoxide at a rate of 300 μmol mg chlorophyll^{-1} h^{-1} ($60\,O_2^-\ P_{700}{}^{-1}s^{-1}$) at maximum. In addition, requirements of oxygen concentration (K_m 100 μM or 8.4 kPa) and of light intensity (300 μmol photons $m^{-2}s^{-1}$ for saturation) are higher than those for thylakoids only (Miyake *et al.* 1998), which meet requirements for the conditions observed in intact leaves and cells (table 3).

Ferredoxin-$NADP^+$ reductase catalyses the photoproduction of superoxide by thylakoids (Goetze & Carpentier 1994; Miyake *et al.* 1998), but only when the enzyme is released from the thylakoid membranes, for example, by active oxygens (Palatnik *et al.* 1997). In healthy leaves this enzyme binds to the thylakoid membranes and does not catalyse the production of superoxide, but are released from the membranes with senescence, which enhance the production of superoxide. At present, MDA reductase is the most likely mediator for the enhanced photoreduction of dioxygen, i.e. enhanced operation of the water–water cycle, because of its higher contents than glutathione reductase in the stroma. The mechanism of how the stromal factor-mediated photoreduction of dioxygen is regulated in chloroplasts and adjusted to the physiological requirements under photon excess conditions remains unknown. Primarily, the competition of the electrons generated in PS I between $NADP^+$ via ferredoxin and the stromal factor determine

the rate. Actually, the PS I-reduced MDA reductase is rapidly auto-oxidized producing superoxide in the presence of NADH, but the auto-oxidation is completely suppressed on addition of NAD^+ (C. Miyake, unpublished data). Furthermore, a putative regulation via the redox state of the intersystem electron carriers is also a possible mechanism.

Bipyridyl derivatives such as paraquat (1,1′-dimethyl-4,4′-bipyridylium chloride) induce the photodamage to leaf tissues by active oxygens derived from the superoxide generated via auto-oxidation of their radicals photoproduced in PS I. The most distinct difference between paraquat- and stromal factor-enhanced photoreductions of dioxygen is the rapid photoreduction rate of paraquat as compared with that of dioxygen in PS I. The photoreduction rate constant of paraquat with PS I is $1.7\times10^8\,M^{-1}s^{-1}$ (Takahashi & Katoh 1984), and the resulting paraquat cation radical is rapidly auto-oxidized to produce superoxide at $7.7\times10^8\,M^{-1}s^{-1}$ (Farrington *et al*. 1973). Because of the high reaction rate, almost all electrons generated in PS I are trapped by paraquat, and few electrons are available for either the associated regeneration of ascorbate in the water–water cycle or the photoreduction of $NADP^+$ via ferredoxin. Thus, ascorbate is not regenerated, and hydrogen peroxide accumulates when endogenous ascorbate is exhausted (Nakano & Asada 1980). In the absence of ascorbate, the reaction intermediate APX compound I is decomposed by hydrogen peroxide resulting in inactivation (Miyake & Asada 1996), and chloroplastic APX, especially the thylakoid-bound form, is inactivated by application of paraquat (Miyagawa *et al*. 2000; J. Mano, unpublished data). This mechanism is why paraquat causes photoinhibition and is used as a herbicide. Microbial toxins like cercosporin and naphthazarins photoproduce superoxide in chloroplasts and induce photodamage (Heiser *et al*. 1998). Similar to paraquat, the production rate of superoxide by these toxins is so high that the associated regeneration of ascorbate could not operate, resulting in the accumulation of hydrogen peroxide.

When superoxide and hydrogen peroxide produced in PS I are not effectively scavenged, the hydroxyl radicals are produced by their interaction with a trace amount of Fe or Cu ions or by the interaction of hydrogen peroxide with the reducing side of PS I (Jakob & Heber 1996). At least in intact thylakoid membranes, the generation of superoxide is limited to the reducing side of PS I, and no superoxide generation has been detected in other possible sites such as the reducing side of PS II (Q_A and Q_B), plastoquinone and the Q-cycle. PS II membranes photoproduce superoxide (Chen *et al*. 1995) but intact thylakoid membranes do not, indicating the protection of reduced Q_A or Q_B from its interaction with dioxygen. In contrast to the reduced species of oxygen, singlet excited oxygen ($^1O_2$) is not photoproduced in PS I even under photon excess conditions (Hideg & Vass 1995). On the contrary, $^1O_2$ is photoproduced in PS II via the interaction of the triplet-excited reaction centre chlorophyll ($^3P^*_{680}$), with triplet ground-state dioxygen ($^3O_2$) when photon intensity is in excess of its use capacity and $^3P^*_{680}$ is produced through recombination in PS II (Hideg *et al*. 1998, this issue).

4. PHYSIOLOGICAL FUNCTIONS OF THE WATER–WATER CYCLE

(a) *Suppression of the oxidation of target molecules by active oxygens*

The water–water cycle allows reduction of dioxygen to water at rapid rates without releasing reactive species of oxygen from the generation site in PS I, i.e. superoxide and hydrogen peroxide disappear prior to their diffusion from the thylakoidal scavenging complex. Even if they leak from the thylakoidal system, the stromal system scavenges them. Therefore, the primary function of the water–water cycle is the protection of chloroplast components from their interaction with active oxygens. For details of the target molecules of active oxygens in chloroplasts, see a recent review by Asada (1999). Oxidation of the target molecules inactivates CO_2 fixation (Calvin cycle enzymes including rubisco), photorespiration (glutamate synthase; Kozaki & Takeba 1996), water–water cycle (APX and SOD), and PS I activity itself—all of them lower the photon-using capacity of chloroplasts and enhance further the photoinhibition. The *de novo* synthesis system of D_1 protein of PS II in chloroplasts for repairing PS II photoinhibition is also sensitive to active oxygens (Nishiyama *et al*. 2000).

(b) *Enhanced photoinihibition by anaerobiosis*

Active oxygens are photoproduced either by the reduction or excitation of dioxygen in chloroplasts—suppression of photo-oxidative damage or enhancement of photosynthesis under low concentrations of oxygen would therefore be expected. However, this is not the case—under low concentrations of oxygen, photoinhibition of thylakoids, chloroplasts, leaf tissues and algal cells is enhanced, especially when photon intensity is in excess of its use capacity. At low (1–2 kPa) oxygen, photorespiration is suppressed due to high K_m-values (O_2) of rubisco and glycolate oxidase, and the CO_2 fixation is rather increased under such mild conditions as high CO_2 concentrations and low light intensity. However, under CO_2-deficient conditions photoinhibition is induced because excess photon energy is not effectively dissipated via photorespiration in low O_2 (Osmond 1981). Furthermore, under anaerobic conditions, enhanced photoinihibition has been repeatedly observed after the first discovery in thylakoids by Trebst (1962). Under such conditions, for thylakoids no electron acceptor is available for PS I and the intersystem electron carriers between PS II and PS I are over-reduced, which induces photoinactivation of PS II. Anaerobiosis-induced photoinhibition has been observed in intact chloroplasts, mesophyll cells, algal cells and leaf tissues (Park *et al*. 1996), as reviewed by Asada (1999). Thus, over several kilopascals of oxygen is required not only for the normal photosynthesis but also for the suppression of photoinhibition.

(c) *Electron flux through the water–water cycle*

As long as the water–water cycle operates effectively in chloroplasts, the reduced species of oxygen do not inactivate the target molecules, as described in § 4(a). Oxygen requirement for photosynthesis and enhanced photoinhibition in anaerobiosis, especially under photon excess conditions, suggest another function of the

water–water cycle. The electron flux rates through the water–water cycle in algae and plants have been deduced under various stress conditions (table 4). Carbon dioxide fixation, $^{18}O_2$ uptake (Badger *et al.*, this issue) and electron flux through PS II, as determined by chlorophyll fluorescence under various light intensities and oxygen concentrations, and a combination of these and other methods, have been used to deduce the contribution of the electrons generated in PS II to CO_2 assimilation (A), photorespiration and the water–water cycle.

All of the data in table 4 do not allow an estimation of the ratio of the electron flux and oxygen concentrations required for the water–water cycle, but the cycle operates for the dissipation of excess electrons at least in algae and C_4 plants. In algal cells the deduced electron flux through the water–water cycle appears to be highest, as discussed above. In C_3 plants, photorespiration makes it difficult to estimate the electron flux through the water–water cycle but the flux appears to increase under conditions of photon excess induced by CO_2 depletion, bright light, drought stress, chilling stress, salt stress and senescence. In tobacco whose rubisco is reduced, no oxygen uptake thought the water–water cycle was observed (Ruuska 2000). In C_4 plants, the absence of oxygen uptake through photorespiration makes it easy to estimate the electron flux through the water–water cycle, and the flux has been observed under environmental stress. The deduced flux rate through the water–water cycle cannot be accounted for by the thylakoid-dependent photoreduction of oxygen but only by the stromal factor-enhanced one, in respect of oxygen concentration and light intensity (table 3). Thus, the linear electron flux through the water–water cycle increases under photon excess conditions and protects from photoinhibition by the following mechanisms as far as the cycle effectively scavenges reactive species of oxygen.

(d) *ATP and ΔpH generation*

The water–water cycle allows linear electron flow and generates a proton gradient across the thylakoid membranes (Schreiber *et al.* 1995), but no net production of reduced ferredoxin and NADPH is expected. First, the properties of the water–water cycle make it possible to adjust promptly the photoproduction ratio of ATP–NADPH required for CO_2 fixation and other stromal reactions—a classical pseudocyclic photophosphorylation. Fine tuning of the production ratio of ATP–NADPH is possible also by the cyclic electron flow around PS I, but the cyclic electron flow operates only under conditions where the electron flow from PS II is limited. For C_3 CO_2 fixation, linear electron flow supplies ATP without the help of either the water–water cycle or cyclic electron flow, but C_4 CO_2 fixation requires more ATP than C_3 plants. It has been shown that 5–10 kPa O_2 is required for the highest CO_2 fixation of C_4 plants (Maroco *et al.* 2000), which supplies additional ATP for CO_2 fixation in the mesophyll cells, which indicates the participation of the stromal factor-enhanced water–water cycle in ATP production. Guard cells require ATP for potassium ion movement in the regulation of stomata functioning. Chlorophyll fluorescence of guard cell chloroplasts is largely affected by anaerobiosis, which suggests that, as a possible mechanism, the water–water cycle is the major electron flux for ATP production (Goh *et al.* 1999). Thus, the water–water cycle is able to reinforce the photon using capacity (A) by ATP supply. Furthermore, ATP required for other chloroplast reactions, such as repairing of the target molecules under photon excess conditions, could be supplied by the water–water cycle. Photorespiration requires more ATP than for the CO_2 fixation cycle, and the water–water cycle could supply additional ATP required for the operation of photorespiration, which also dissipates excess photon energy.

The water–water cycle itself does not consume any ATP and effectively generates a proton gradient across the thylakoid membranes (Schreiber *et al.* 1995). Downregulation of the quantum yield of PS II is a rapid response to the photon excess conditions and requires a proton gradient. When the electron acceptor in PS I is not available, for example under conditions of limited CO_2 supply to chloroplasts, little electron flux from PS II to PS I is expected to generate the proton gradient. Under such conditions, the water–water cycle could promptly generate a proton gradient across the thylakoid membranes and trigger the downregulation of PS II to dissipate excess photon energy.

(e) *Dissipation of excess photon energy*

When PPFD is in excess of that required to support the rate of CO_2 fixation (A), the downregulation of PS II is an effective way to dissipate rapidly the excess photon energy. Decrease of the quantum yield of PS II by the proton gradient-dependent downregulation has been estimated to be between 60 and 35%, as determined by non-photochemical quenching of chlorophyll fluorescence under field conditions where little irreversible photoinhibition is induced (Genty & Harbinson 1996). Furthermore, it has been suggested that the lowest pH in the lumen at high PPFD, up to 3000 $\mu mol\,m^{-2}\,s^{-1}$, is regulated to maintain between 5.8 and 6.5, as determined by the kinetic half-lives of re-reduction of cytochrome f in chloroplasts of intact leaves. Under extreme photon excess conditions, where the lumen pH is below pH 5.5, the donor side of PS II is inactivated and plastocyanin is acid decomposed, resulting in irreversible photoinhibition (Kramer *et al.* 1999). Thus, an upper limit of the dissipation of excess photons as heat via the downregulation of PS II is around one-half of the absorbed photons. When excess photon energy cannot only be dissipated by downregulation of PS II via the proton gradient, the excess electrons generated in PS II could be dissipated either by photorespiration or the water–water cycle. In C_4 plants the water–water cycle could dissipate excess photons and electrons under photon excess stress, since little contribution of photorespiration to the dissipation is expected.

5. FUNCTIONAL EVOLUTION OF THE WATER–WATER CYCLE

As described above, the water–water cycle of plants has, at least, two physiological functions to suppress the photoinhibition when photon intensity is in excess of that required to support the photon using capacity, CO_2 assimilation (A); first, the rapid scavenging of active oxygens, and second, dissipation of and relaxation from

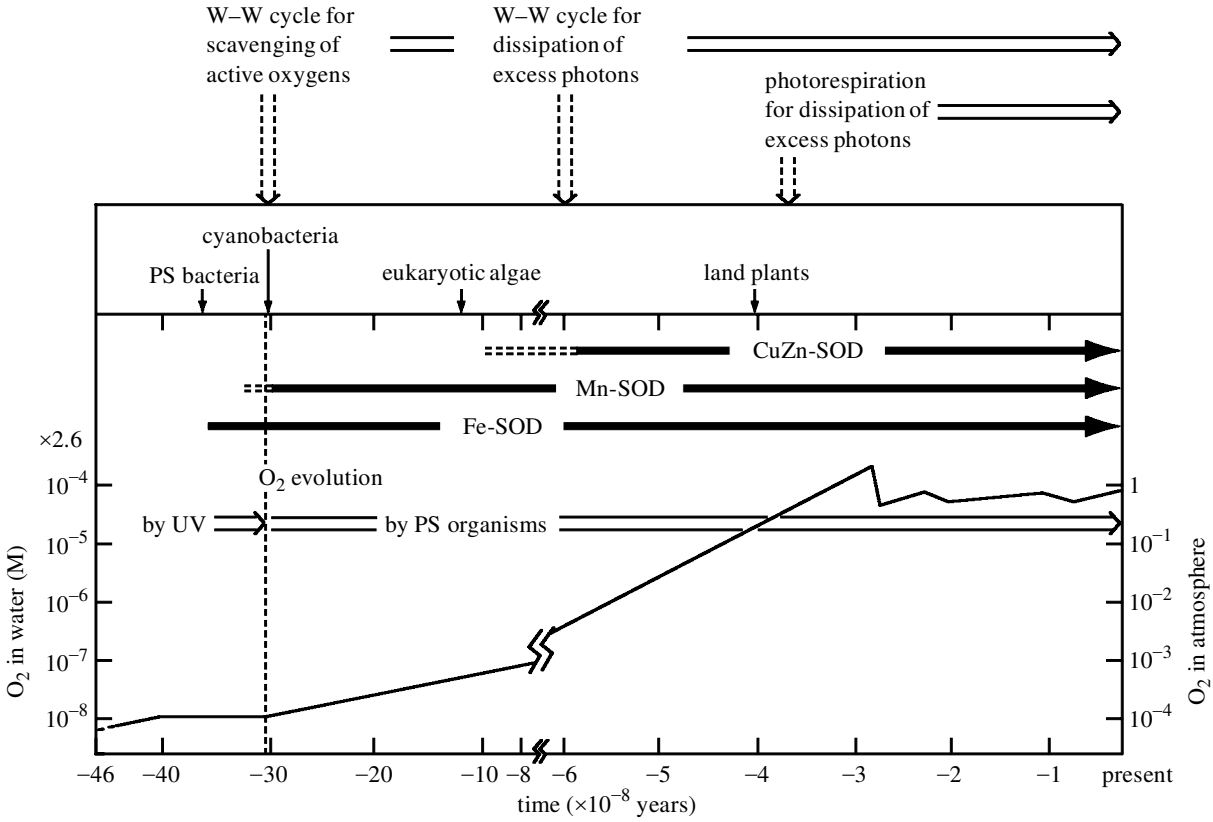

Figure 2. Functional evolution of the water–water (W–W) cycle in photosynthetic (PS) organisms accompanied by an increase in the concentration of atmospheric dioxygen on the Earth. Right ordinate, represents oxygen concentration relative to present atmospheric level. Left ordinate, concentration in water equilibrated with the atmosphere at 25 °C. Acquisition of Fe-SOD, Mn-SOD and Cu,Zn-SOD, accompanied by an increase of atmospoheric dioxygen concentration, is included (Asada *et al.* 1980).

excess photon stress. Occurrence of Fe-SOD in the anaerobic photosynthetic bacteria *Chlorobium* and *Chromatium* (Kanematsu & Asada 1978, 1979) indicates that the production of active oxygens was unavoidable even under extremely low concentrations of dioxygen when they appeared on the Earth over 3×10^9 years ago. The oxygen concentration then has been estimated to be 10^{-4} of present atmospheric levels (2.1 Pa, 2.6 nM in water at 25 °C), and even under such conditions rapid scavenging of active oxygens would have been essential to avoid photo-oxidative damage. Mn-SOD was acquired followed by Fe-SOD, and then Cu,Zn-SOD, accompanied by an increase in atmospheric concentration of oxygen (Asada *et al.* 1980) (figure 2).

APX activity has been found in cyanobacteria (Tel-or *et al.* 1986; Miyake *et al.* 1991; Miller *et al.* 2000), but we failed to detect ascorbate in cyanobacteria as in other prokaryotes. However, several species of cyanobacteria, including *Synechocystis*, show the stoichiometric photoevolution of $^{16}O_2$ on addition of $H_2{}^{18}O_2$ as observed in spinach chloroplasts—indicating the reduction of photoproduced hydrogen peroxide to water by the photoreductants via a peroxidase reaction. Other species of cyanobacteria disproportionate hydrogen peroxide to dioxygen and water by catalase as observed by $^{18}O_2$ evolution on addition of $H_2{}^{18}O_2$ in both dark and light (Miyake *et al.* 1991). In cyanobacteria catalase–peroxidase has been characterized (Mutsuda *et al.* 1996). Thus, the peroxidase scavenging system of hydrogen peroxide was acquired during the evolution of cyanobacteria. Recently it has been shown that thioredoxin peroxidase (2-Cys peroxiredoxin) plays a role as a hydrogen peroxide-scavenging peroxidase in the prototype of the water–water cycle in *Synechocystis* (Yamamoto *et al.* 1999). 2-Cys peroxiredoxin also plays a role in the protection from oxidative stress in plant chloroplasts (Baier & Dietz 1999). Since occurrences of APX, ascorbate and the operation of the water–water cycle have been shown in *Chlamydomonas* and *Euglena* (Miyake *et al.* 1991), the water–water cycle of figure 1 seems also to be functional in eukaryotic algae in addition to land plants.

While the atmospheric oxygen concentration was below 1 kPa, the physiological function of the water–water cycle would be limited to the scavenging of active oxygens photoproduced by PS I of the thylakoids. Since the stromal factor-enhanced production of superoxide requires higher concentrations of oxygen than that by thylakoids only (table 3), the water–water cycle was employed by organisms for the dissipation of excess photons after the atmospheric concentrations of oxygen increased to at least 1 kPa, although its function did not operate at maximum level (figure 2). Since photorespiration requires higher concentrations of oxygen than that for the stromal factor-enhanced photoreduction of oxygen, it is reasonable to assume that the dissipation mechanism of excess photon energy via photorespiration was acquired by plants following that via the water–water cycle.

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Discussion

J. Allen (*Department of Plant Cell Biology, Lund University, Sweden*). What is the electron donor to MDAR?

K. Asada. The photoreduction of the FAD-enzyme MDAR, as determined by the bleaching of the FAD in absorbance at 450 nm, was observed by washed thylakoids, and it was inhibited by either DCMU or DBMIB. Since this photoreduction was not enhanced by the addition of either ferredoxin or ferredoxin-$NADP^+$ reductase, then I suppose that the FAD of MDAR is directly photoreduced by the reducing side of PS I, possibly by the centre A/B.

J. Allen. Does water–water cycle operate from water $\rightarrow$ PS II $\rightarrow$ PS I $\rightarrow$ MDAR $\rightarrow O_2$?

K. Asada. Under the conditions where chloroplasts should dissipate a large excess electrons, the MDAR-mediated photoreduction of dioxygen would be the major route in the water–water cycle. However, a mechanism to regulate the rate of photoreduction of oxygen depending on the amounts of excess electrons remains to be revealed.

J. Allen. Is the donor P_{700}?

K. Asada. I suppose that P_{700} is not a direct electron donor to MDAR, because the interaction of P_{700} with MDAR (molecular size of 47 kDa) is unlikely to occur in the thylakoids.

J. Allen. Ferredoxin reduces O_2 at high rates—is it the factor required for O_2 uptake from the thylakoids?

K. Asada. Ferredoxin is not required for the photoreduction of MDAR. Auto-oxidation rate of reduced ferredoxin is not so high as compared with that of reduced MDAR. Furthermore, reduced ferredoxin is oxidized rapidly by the monodehydroascorbate radical produced by the ascorbate peroxidase reaction at a similar rate to that of the auto-oxidation of MDAR (around $10^7\,M^{-1}s^{-1}$). Thus, in chloroplasts the auto-oxidation of reduced ferredoxin would not be the major source of superoxide generation.

C. H. Foyer (*Department of Biochemistry and Physiology, IACR-Rothamsted, UK*). The role of superoxide dismutase has always been difficult to understand because the enzyme-catalysed reaction is only slightly faster than the chemical reaction. Furthermore, it cannot be a rate-limiting reaction but over-expression of SOD leads to enhanced protection of photosynthesis. Your data show that Cu,Zn-SOD antisense plants have a shrunken phenotype and show inhibition of PS I. I would suggest that this is a reflection of the role of superoxide dismutase in substrate channelling rather than simple catalysis. Hence, the function of Cu,Zn-SOD is to channel superoxide to H_2O_2 formation close to the thylakoid bound ascorbate peroxidase. This channelling of superoxide and H_2O_2 would limit the possibility of oxidative damage.

K. Asada. In chloroplasts, Cu,Zn-SOD is compartmentalized or attached on the PS I complex where superoxide is generated, and its local concentration on the thin layer (5 nm) on the thylakoid membranes is nearly 1 mM. Since the reaction rate constant of superoxide with SOD ($2\times10^9\,M^{-1}s^{-1}$) is four orders of magnitude higher than that with ascorbate ($3\times10^5\,M^{-1}s^{-1}$), the simulated disproportionation of superoxide by SOD is over 99% from their concentrations (ascorbate, 30 mM; Cu,Zn-SOD, 1 mM). Therefore, the attachment of the Cu,Zn-SOD on the thylakoid membranes allows the channelling of the superoxide to the thylakoid-bound ascorbate peroxidase, rather than its interaction with ascorbate. If Cu,Zn-SOD is distributed uniformly in chloroplasts, its concentration is lowered to around 10 μM, and the interaction of superoxide with ascorbate or glutathione increased to a similar order to that with SOD, which causes the photooxidative damage.

C. H. Foyer. Do you have any data on the activity and role of thylakoid-bound Fe-SOD in the Cu,Zn-SOD antisense plants? Evidently Fe-SOD cannot compensate for low Cu,Zn-SOD but is expression of the protein increased in the Cu,Zn-SOD antisense transformants?

K. Asada. According to preliminary assays, the Cu,Zn-SOD antisense tobacco had about 18% Cu,Zn-SOD of the wild-type, but the contents of Fe-SOD of the Cu,Zn SOD antisense plants are about 50–60% that of the wild-type. In wild-type the activity ratios of the two types of SOD are similar to each other. Intrachloroplastic localization of Fe-SOD is not determined, but Fe-SOD appears not to compensate for a low activity of the thylakoid-attached Cu,Zn-SOD.

H. C. P. Matthijs (*Department of Microbiology, University of Amsterdam, The Netherlands*). I wish to ask your opinion on the distribution of cyclic electron flow and the

water–water cycle in photosynthetic organisms: the water–water pathway aids ATP formation, and so does PS I cyclic electron transfer. In your figure 2 you showed that the water–water pathway was less effective in cyanobacteria than in plant chloroplasts. How would you see the use of PS I cyclic versus water–water in evolutionary terms? In what way do you compensate for cytochrome aa_3 contribution?

K. Asada. When the cyanobacteria appeared over 3×10^9 years ago, the atmospheric concentration of oxygen was extremely low (10^{-4} of the present level). Then the cytosol factor-mediated water–water cycle could not operate, because it requires 5–10% of atmospheric oxygen for half saturation. However, the cyclic electron flow around PS I is possible even under these conditions. Therefore, I suppose that the water–water cycle for the dissipation of excess electrons could not operate in cyanobacteria when they appeared, and its operation started after the accumulation of oxygen in the atmosphere. Both the water–water cycle and the cyclic electron flow generate ΔpH across the thylakoid membranes and ATP without generation of biochemical reductants, but the operation of the cyclic electron flow around PS I is limited only when the electron supply from PS II is limited or the molar ratio of PS I is higher than that of PS II under white light. Under the specific conditions where the cells are illuminated by far-red light, the cyclic electron flow around PS I would be the major flux.

In cyanobacteria, generally the PS I complex is rich as compared with the PS II complex, and the cyclic electron flow would be major flux when either ΔpH or ATP is required. In plant chloroplasts, except for those of the bundle sheath cells of C_4 plants, the cyclic flow around PS I is very limited, at least prior to the downregulation of PS II. For the downregulation of PS II, the water–water cycle is necessary to trigger the ΔpH generation. Even after the PS II yield is downregulated by ΔpH, it decreases only to one-half. Under the conditions where the remaining half of the electrons from PS II should be dissipated, the water–water cycle is necessary in addition to the cyclic electron flow. Chlororespiration connected to the putative thylakoid-bound cytochrome oxidase would not be so high as long as PS I is excited.

H. C. P. Matthijs. Once again, what is the relative role of the water to water cycle versus PS I cyclic?

K. Asada. As stated above, the molar ratio of PS I–PS II, downregulation of PS II and excitation ratio of PS I–PS II depending on the light spectrum would determine the operation ratio of the water–water cycle and cyclic electron flow, when chloroplasts are exposed to light intensities in excess of that required for CO_2 fixation.