Production and Scavenging of Reactive Oxygen Species in Chloroplasts and Their Functions[1]

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The reaction centers of PSI and PSII in chloroplast thylakoids are the major generation site of reactive oxygen species (ROS). Photoreduction of oxygen to hydrogen peroxide (H_2O_2) in PSI was discovered over 50 years ago by Mehler (1951). Subsequently, the primary reduced product was identified to be superoxide anion (O_2^-), and its disproportionation produces H_2O_2 and O_2 (Asada et al., 1974). On the other hand, in PSII, oxygen of the ground (triplet) state ($^3O_2$) is excited to singlet state ($^1O_2$) by the reaction center chlorophyll (Chl) of triplet excited state ($^3P680*$; Telfer et al., 1994; Hideg et al., 1998).
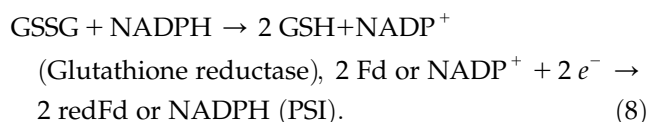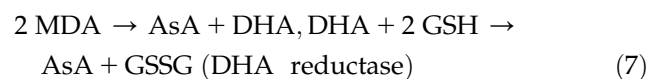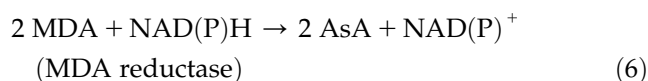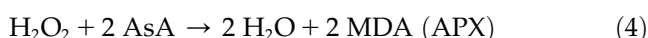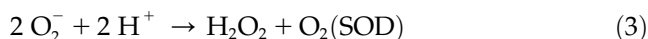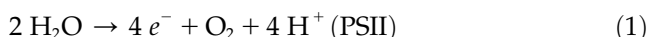
The photoproduction of ROS is largely affected by physiological and environmental factors; the rate is enhanced under the conditions where photon intensity (P) is in excess of that required for the CO_2 assimilation (A). Under the conditions of photon excess ($P > A$), the relaxation systems suppress the photoproduction of ROS in chloroplasts, such as photorespiration, the cyclic electron flows through either PSI or PSII, and the down-regulation of PSII quantum yield as regulated by the xanthophyll cycle and the proton gradient across thylakoid membrane. Prompt scavenging of the ROS produced in thylakoids prior to its diffusion from the generation site is indispensable to protect the target molecules in thylakoid and stroma.

Here, the production of reduced and excited species of ROS and their scavenging system in chloroplasts are overviewed. The photoproduction and subsequent scavenging of ROS not only protect chloroplasts from the direct effects of ROS, but also relax the photon (electron) excess stress, and these physiological functions of ROS production and scavenging are discussed.

PHOTOREDUCTION OF OXYGEN IN PSI TO WATER VIA O_2^- AND H_2O_2: WATER-WATER CYCLE

In thylakoids, H_2O_2 is photoproduced via O_2^- and accumulates, but in intact chloroplasts, H_2O_2 does not accumulate. Localization of ascorbate peroxidase (APX) and related enzymes indicates that chloroplasts reduce H_2O_2 with APX using the electrons derived from water in PSII as follows:

$$2 H_2O \rightarrow 4\,e^- + O_2 + 4 H^+ \text{ (PSII)} \tag{1}$$

$$2 O_2 + 2\,e^- \rightarrow 2 O_2^- \text{ (PSI)} \tag{2}$$

$$2 O_2^- + 2 H^+ \rightarrow H_2O_2 + O_2 \text{(SOD)} \tag{3}$$

$$H_2O_2 + 2 AsA \rightarrow 2 H_2O + 2 MDA \text{ (APX)} \tag{4}$$

$$2 MDA + 2 redFd \rightarrow 2 AsA + 2 Fd \text{(spontaneous)} \tag{5}$$

$$2 MDA + NAD(P)H \rightarrow 2 AsA + NAD(P)^+$$
$$\text{(MDA reductase)} \tag{6}$$

$$2 MDA \rightarrow AsA + DHA, DHA + 2 GSH \rightarrow$$
$$AsA + GSSG \text{ (DHA reductase)} \tag{7}$$

$$GSSG + NADPH \rightarrow 2 GSH + NADP^+$$
$$\text{(Glutathione reductase), } 2 Fd \text{ or } NADP^+ + 2\,e^- \rightarrow$$
$$2 redFd \text{ or } NADPH \text{ (PSI).} \tag{8}$$

The primary product of oxygen reduction, O_2^- (Eq. 2), is disproportionated to H_2O_2 and O_2 catalyzed with superoxide dismutase (SOD; Eq. 3). The H_2O_2 generated by SOD is reduced to water by ascorbate (AsA) catalyzed with APX, and AsA is oxidized to monodehydroascorbate radical (MDA; Eq. 4). Subsequently, MDA is directly reduced to AsA by either reduced ferredoxin (redFd; Miyake and Asada, 1994; Eq. 5) or NAD(P)H catalyzed with chloroplastic MDA reductase (Sano et al., 2005; Eq. 6). If MDA fails to reduce directly to AsA, it is spontaneously disproportionated to dehydroascorbate (DHA) and AsA. DHA is then reduced to AsA by reduced glutathione (GSH) catalyzed with DHA reductase (Shimaoka et al., 2003; Eq. 7). Finally, Fd and $NADP^+$ for the regeneration of AsA (Eqs. 5–7) are reduced in PSI (Eq. 8). Thus, in any pathways of the regeneration of AsA, the half electrons derived from water in PSII (Eq. 1) are used for the univalent reduction of oxygen (Eq. 2) and another half for the generation of reductants (Eq. 8) to reduce H_2O_2 (Eqs. 4–7), which has been referred to as the water-water (W-W) cycle.

PARTICIPATING ENZYMES IN THE W-W CYCLE

SOD

Several plants contain Fe-SOD in addition to CuZn-SOD, but no Mn-SOD, in chloroplasts. In anaerobic

bacteria, the reduced form of iron-sulfur protein, such as rubredoxin and neelaredoxin, can reduce O_2^- to H_2O_2 (Abreu et al., 2001), but in chloroplasts no such iron-sulfur proteins are found.

APX

APX is classified as class I peroxidase similar to Cyt c peroxidase (CcP) and is different from class III peroxidase such as horseradish (*Armoracia lapathifolia*) peroxidase (Raven, 2003). Even though CcP and APX are classified in the same family, compound I of CcP is stable, but that of APX is not so stable in the absence of AsA (Miyake and Asada, 1996). Compound I of chloroplastic APX is more labile as compared with that of cytosolic APX (Amako et al., 1994). In contrast to plant APX, compound I of APX from a red alga is stable (Sano et al., 2001). APX is inhibited by thiol-modifying reagents, and the Cys residue participates in the binding of AsA to the heme (Raven, 2003).

Chloroplastic APX is classified into thylakoid-bound (tAPX) and stroma-localized forms. tAPX binds in the vicinity of PSI (Miyake et al., 1993), and its anchor is the C-terminal, hydrophobic 50 residues. Except for Arabidopsis (*Arabidopsis thaliana*), a single gene codes tAPX and stroma-localized APX, and both isoforms are generated by alternative splicing (Yoshimura et al., 2002).

Peroxiredoxin

Prokaryotes lack AsA and cyanobacteria scavenge H_2O_2 with the thioredoxin-peroxiredoxin (Prx) system (Yamamoto et al., 1999). Thioredoxin and Prx are localized in plant chloroplasts, but it is not known under what conditions this system functions as an alternative W-W cycle in place of APX. Prx Q associates to PSII complexes (Lamkemeyer et al., 2006).

MDA Reductase

In addition to MDA, MDA reductase is able to reduce phenoxyl radicals, such as quercetin radicals to their parent phenols (Sakihama et al., 2000). Thus, this enzyme would participate also in the regeneration of antioxidants. In Arabidopsis, MDA reductase (Obara et al., 2001), stromal APX, and glutathione reductase gene products (Chew et al., 2003) have dual targeting into chloroplasts and mitochondria.

Catalase

In chloroplast stroma, no catalase has been found. However, PSII membranes associate a heme catalase (Sheptovitsky and Brudig, 1996). This catalase would not directly participate in the W-W cycle but protects water oxidase in the lumen if the W-W cycle does not operate properly and H_2O_2 diffuses to the lumen.

A-Type Flavoprotein

This protein participates in the photoreduction of O_2 in *Synechocystis* PCC6803, catalyzing the four electron reduction of O_2 to $2 H_2O$ using $2 NAD(P)H$ without releasing ROS (Helman et al., 2005).

THYLAKOIDAL AND STROMAL SCAVENGING SYSTEMS OF ROS IN CHLOROPLASTS

In chloroplasts, over one-half of CuZn-SOD attaches on the stroma thylakoids where PSI is localized (Ogawa et al., 1995). PSI-attached SOD, tAPX bound to in the vicinity of PSI and Fd-dependent reduction of MDA form the thylakoidal scavenging system (Eqs. 3–5), which functions as the first defense against ROS (Fig. 1). Other scavenging enzymes would be compartmented in the stroma and play a role as the second defense (stromal scavenging system). In the thylakoidal system, local concentrations of SOD and tAPX of around 1 mM in the vicinity of PSI and respective reaction rates of the participating enzymes allow us to estimate the scavenging rates of O_2^- and H_2O_2 and their steady-state concentrations (Asada, 1999, 2000; Polle, 2001). These simulations indicate that the limiting step of the W-W cycle is the reduction of oxygen (Eq. 2), and its rate is lower at least two orders of magnitude than those of the SOD-catalyzed disproportionation of O_2^- (Eq. 3), the reduction of H_2O_2 by APX (Eq. 4), and the reduction of MDA (Eq. 5). Therefore, the total electron flux through the W-W cycle is spontaneously twice the rate of Equation 2. In the presence of methyl viologen (MV) the rate of Equation 2 is higher due to rapid autooxidation of the photoreduced MV radicals to form O_2^- in PSI, then few electrons are available for Equation 8 to regenerate AsA, and H_2O_2 cannot be reduced. Under such conditions, APX is rapidly inactivated due to decomposition of the compound I, and oxidative damages are amplified (Mano et al., 2001).

In chloroplasts, AsA is rapidly regenerated from either MDA or DHA (Eqs. 5–8). In addition to the electron donors for APX, AsA participates in the following reactions: the cofactor for violaxanthin deepoxidase in the xanthophyll cycle, acute electron donors to PSI and PSII in the lumen (Mano et al., 2004), maintenance of the cyclic electron flow as the compensatory electron donor (Ivanov et al., 2005), and regeneration of tocopherols from its oxidized radicals to suppress lipid oxidation in thylakoids (Munne-Bosch and Alegre, 2002).

INCREASED ELECTRON FLUX THROUGH W-W CYCLE UNDER *P* > *A* CONDITIONS

The electron flux through the W-W cycle was estimated from CO_2 assimilation and parameters of Chl fluorescence or $^{18}O_2$ uptake. The flux through the W-W cycle of dark-adapted leaves (Makino et al., 2002) and algal cells (Radmer and Kok, 1976) prior to the
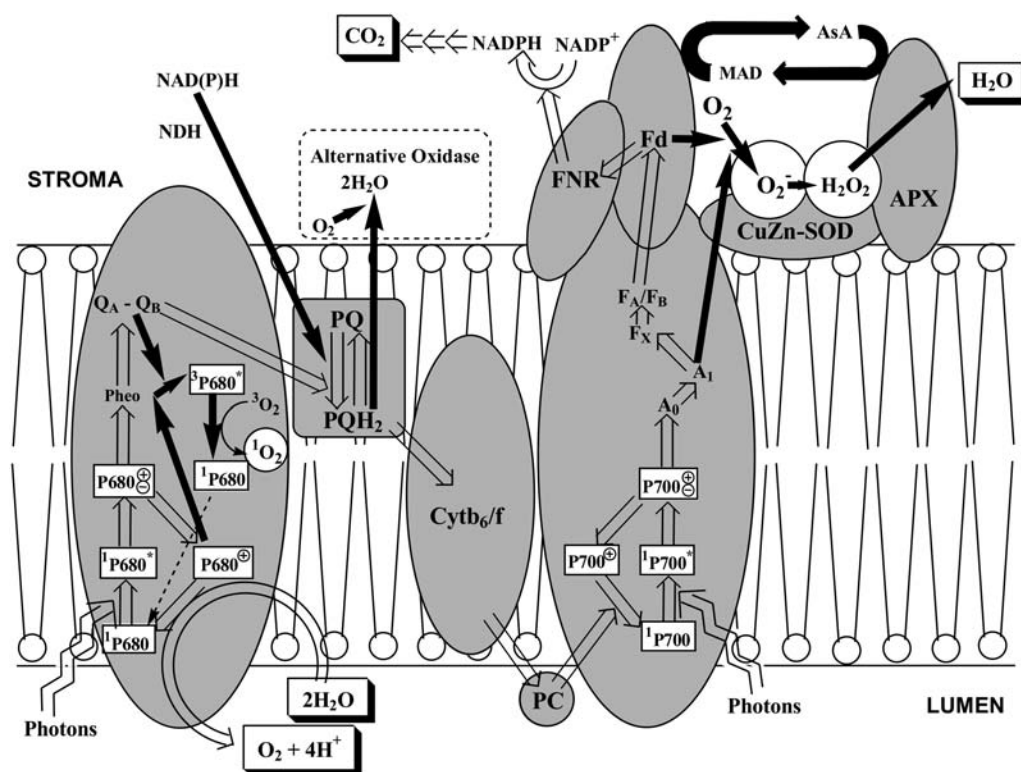
Figure 1. Productions of $^1O_2$ in PSII and of O_2^- in PSI in chloroplast thylakoids. White arrows represent the photoexcitation of reaction center Chl and the electron flow under the photon intensity where all of the electrons generated are utilized for the CO_2 assimilation ($P < A$ or $P = A$). Black arrows represent where photon intensity exceeds the capacity of the flux of electron transport and the flux to CO_2 assimilation ($P > A$). To make it simple, the stromal scavenging system of ROS is omitted and the thylakoidal scavenging system only is shown. Alternative oxidase is a four-electron oxidase using plastoquinol as the electron donor ($2\ PQH_2 + O_2 \rightarrow 2\ PQ + 2\ H_2O$) without releasing ROS and functions in chlororespiration in the dark (Aluru and Rodermel, 2004). NDH, NAD(P)H dehydrogenase complex; PQ, plastoquinone; PQH_2, plastoquinol.

photoactivation of the Calvin cycle is very similar to that for the CO_2 assimilation at steady state after the photoactivation. These observations indicate that the capacity of the W-W cycle is high enough to allow the electron flux at the steady state of photosynthesis, and prior to the photoactivation, the W-W cycle is the major alternative pathway. In this respect, the W-W cycle appears to be indispensable to start photosynthesis of dark-adapted leaves without photooxidative damages. In maize (*Zea mays*), oxygen at low concentration (half saturation; 0.15 kPa) is required to initiate CO_2 assimilation (T. Ohwaki, K. Asada, unpublished data).

A similar increase in the electron flux through the W-W cycle has been observed when the CO_2 assimilation (*A*) is suppressed by CO_2-limiting conditions (Miyake and Yokota, 2000), low temperatures (Hirotsu et al., 2004; Li et al., 2005), and salt stress (Chen et al., 2004).

GENETIC MODIFICATION OF W-W CYCLE ENZYMES

The key enzymes in the thylakoidal scavenging system, tAPX and chloroplastic CuZn-SOD, have genetically altered. Mutants of tAPX are thought to be lethal (Yabuta et al., 2002). Plants with reduced tAPX

activity are sensitive to MV stress (Tarantino et al., 2005), and show decreases in PSII activity, CO_2 assimilation, and biomass production (Danna et al., 2003). Phenotypes of knockdown mutant of chloroplastic CuZn-SOD are suppressed growth, small chloroplasts, and low photosynthetic activity (Rizhsky et al., 2003). These phenotypes confirm that the thylakoidal scavenging system of ROS is essential for photosynthesis even under mild conditions. A low scavenging capacity of ROS in the vicinity of PSI, for example, during the photoactivation of the Calvin cycle, would inactivate several enzymes for CO_2 assmilation. On the contrary, plants overexpressing tAPX (Yabuta et al., 2002; Murgia et al., 2004) are tolerant to MV stress. When *Escherichia coli* catalase is overexpressed in chloroplasts of tobacco (*Nicotiana tabacum*), plants show enhanced resistance to photooxidative stress by MV (Miyagawa et al., 2000). Because chloroplastic APX is labile as compared with the cytosolic one, cytosolic APX was overexpressed in chloroplasts. Such plants show enhanced tolerance to salt and drought stresses (Badawi et al., 2004). Transformants overexpressing a stable red algal APX (Sano et al., 2001) effectively scavenge H_2O_2 even under conditions where CO_2 assimilation is limited (Miyake et al., 2006). The knockout

mutant of the cytosolic APX disturbs the chloroplastic scavenging system and enhances H_2O_2 accumulation in leaves, indicating that the cytosolic APX is functional in ROS signaling (Davletova et al., 2005).

PHOTOPRODUCTION OF $^1O_2$ IN CHLOROPLASTS

PSII Reaction Center (P680)

In contrast to P700, the life time of $P680^+$ is very short, because of rapid withdrawal of electrons from water to $P680^+$ and the charge recombination of $P680^+$ with the primary electron acceptors of PSII pheophytin, Q_A, and Q_B to form $^3P680^*$, especially when the intersystem electron carriers are reduced. Such a situation is likely to occur under the conditions of $P > A$, where either light intensity is too high or the CO_2-assimilation rate is low due to either environmental stresses or physiological conditions. In illuminated PSII reaction center, $^3P680^*$ was produced under anaerobic conditions, but on addition of $^3O_2$ it decayed to $^1P680$, accompanied with the generation of $^1O_2$, as detected by luminescence at 1,270 nm or a chemical trapping (Macpherson et al., 1993; Telfer et al., 1994): $^3O_2 + {}^3P680^* \rightarrow {}^1O_2 + {}^1P680$ (Fig. 1). In leaf tissues, photoproduction of $^1O_2$ in chloroplasts has been demonstrated using the fluorescence and spin probe DanePy. The yield of $^1O_2$ increases, accompanied by the photoinhibition of PSII by high light or UV (Hideg et al., 2002).

Using another fluorescence probe of $^1O_2$, DPAX (Umezawa et al., 1999), a real-time detection system of $^1O_2$ in illuminated intact thylakoids and chloroplasts was established. In this assay, $^1O_2$ was generated just after the start of the actinic illumination prior to photoinhibition. Under anaerobic conditions, the production rate of $^1O_2$ is immediately enhanced by 5- to 6-fold (E. Yamamoto, N. Umezawa, T. Nagano, Y. Urano, and K. Asada, unpublished data). The similar increase in production rate of $^1O_2$ under anaerobic state was observed using DanePy as a fluorescence probe of $^1O_2$ (K. Asada and E. Hideg, unpublished data).

Enhanced production of $^1O_2$ in thylakoids under anaerobic state is different from that in isolated PSII reaction centers where under anaerobic conditions $^1O_2$ is not photoproduced but only $^3P680^*$. In thylakoids, oxygen functions as the electron acceptor in PSI; therefore, under anaerobic state, the electron flux through the intersystem is suppressed, and the recombination to form $^3P680^*$ in PSII is likely to occur. The source of oxygen for $^1O_2$ generation would be the $^3O_2$ evolved by the water oxidase in the lumen. The anaerobiosis-induced generation of $^1O_2$, however, disappears on addition of the electron acceptor ferricyanide, which confirms that an increased production of $^1O_2$ by $^3P680^*$ is caused by the overreduction of the intersystem carriers.

Anaerobiosis-induced photoinhibition in thylakoids (Trebst, 1962) and Euglena cells (Asada and Takahashi, 1987) has been observed. These paradoxical observa-tions in the respect of photoinhibition by ROS can be inferred by the enhanced photoproduction of $^1O_2$ in PSII under anaerobic state. In other words, the oxygen-dependent electron flux to PSI including the W-W cycle would suppress the generation of $^1O_2$ in PSII and protect the photoinactivation of PSII.

Biosynthetic and Catabolic Intermediates of Chl

Biosynthetic and catabolic intermediates of Chl are photosensitizers to generate $^1O_2$. The catabolic enzyme-deficient and pheophorbide a-accumulating mutant is sensitive to light after dark treatment (Tanaka et al., 2003; Pruzinska et al., 2005). Similarly, the biosynthetic intermediate of Chl, protochlorophillide-accumulating mutant, is also sensitive to light (Wagner et al., 2004).

QUENCHERS AND SCAVENGERS OF $^1O_2$, AND CYCLIC ELECTRON FLOW THROUGH PSII

Since $^1O_2$ is rapidly quenched by water, its life time and diffusion distance from the generation site are very short: 3.1 to 3.9 μs and 190 nm, respectively. In chloroplast thylakoids, the diffusion distance is further shortened because of a higher viscosity and is estimated to be 5.5 nm (Krasnovsky, 1998). Thus, the diffusion distance of $^1O_2$ is the shortest among ROS. For estimation of its biological effects, then, the distances among the generation site of $^1O_2$, quenchers, and target are a critical factor.

In PSII reaction center and antenna subunit complex 11 molecules of β-carotene have been assigned. In the reaction center core, two molecules of β-carotene participate in the quenching of $^1O_2$ generated via $^3P680^*$. Distance between β-carotene and P680 in PSII (Loll et al., 2005) does not allow the quenching of $^3P680^*$ by the β-carotene (for review, see Telfer, 2002). Oxidation of the β-carotene in D_2 to its cation radical has been demonstrated, indicating this β-carotene participates in the cyclic electron flow with Cyt b-559 in PSII (Miyake et al., 2002; Telfer et al., 2003). This cyclic electron transfer through PSII would suppress the generation of $^3P680^*$ and then $^1O_2$ under the conditions of $P > A$.

Tocopherols can quench $^1O_2$, but its rate ($3 \times 10^8 \, M^{-1} \, s^{-1}$) is two orders of magnitude lower than that with β-carotene (Krasnovsky, 1998). α-Tocopherol is oxidized by $^1O_2$ to α-tocopherol quinone via 8-hydroxy-peroxy-tocopherol (for review, see Trebst, 2003), but it is not known by what way α-tocopherol is regenerated. Tocopherols also play a role as an antioxidant to suppress the lipid peroxidation in thylakoids by trapping of lipid radicals (Munne-Bosch and Alegre, 2002).

CONCLUDING REMARKS

Photoproduction of reduced and excited species of ROS in PSI and PSII reaction centers, respectively, is enhanced under the conditions where P is in excess of A. Even under the conditions of $P > A$, where P is too

high or A is low by either environmental stress or physiological state such as prior to the photoactivation of the Calvin cycle, rapid scavenging of ROS by the W-W cycle protects chloroplasts from the direct action of ROS. Further, as long as the W-W cycle operates properly and ROS is promptly scavenged prior to its diffusion to stromal targets, the W-W cycle works as an alternative electron flux and can down-regulate PSII quantum yield by the generation of the proton gradient across the thylakoid membrane. Thus, the W-W cycle functions also as a relaxation system to suppress the photoproduction of $^1O_2$ in PSII.

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