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Biphasic regulation of superoxide radical levels in Mn-depleted and photoactivated photosystem II

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Abstract

In the past decades, it has become clear that superoxide radical ($O_2^{\cdot-}$) can be generated from photosystem II (PSII) during photosynthesis. Depending on the extent of its accumulation, $O_2^{\cdot-}$ plays an important role in plant physiology and pathology. The photoinhibition/repair cycle is a typical process in PSII which is mainly responsible for the survival of plants under the photoinihibition condition. It is therefore of significant importance to determine $O_2^{\cdot-}$ production in this cycle, and then explore how $O_2^{\cdot-}$ is controlled by PSII within a normal physiological level. With this in mind, we herein investigate the variation of the $O_2^{\cdot-}$ levels in PSII under Mn-depleted and photoactivated conditions mimicking the photoinhibition/repair cycle *in vitro*. The effect of intrinsic SOD-like component on the $O_2^{\cdot-}$ levels was also studied. Results show that PSII has the ability to regulate the $O_2^{\cdot-}$ levels in these two processes by simultaneously modulating the $O_2^{\cdot-}$ generation activity and intrinsic SOD-like activity. This finding could shed new lights on the photoprotective property of PSII against $O_2^{\cdot-}$ and other reactive oxygen species.

Additional key words: biphasic regulation; cytochrome b_{559}; Mn-depletion; photoactivation; photosystem II; superoxide radical.

Introduction

Photosystem II (PSII) is a homodimeric protein-cofactor complex embedded in the thylakoid membrane which catalyzes the light-driven charge separation, accompanied by the water-splitting reaction during oxygenic photosynthesis (for reviews *see* Pospíšil 2009 and Müh *et al.* 2008) (Fig. 1). Photodamage to PSII is an unavoidable reaction in photosynthesis (Nishiyama *et al.* 2006). Excitation of the manganese (Mn) cluster with UV or visible light may lead to its inactivation and further release of an excited Mn from the cluster (Tyystjärvi 2008). The Mn release to the lumen leads to the degradation of D1 and relevant proteins, the inhibition of oxygen evolution activity (OEA), as well as the loss of electron transfer activity (Hakala *et al.* 2005). In addition, it has been suggested that both acceptor- and donor-side photoinhibitions lead to the photoinduced damage of PSII (Callahan *et al.* 1986, Vass *et al.* 1992, Vass and Cser 2009). To reduce or prevent the accumulation of dysfunctional PSII, photosynthetic organisms evolve a repairing mechanism mainly involving three steps: degrading the photodamaged D1 protein, resynthesizing this protein (Melis 1999, Aro *et al.* 1993) and photoactivating the inactive Mn-cluster *via* light-driven incorporation of Mn ions into the Mn-depleted cluster (Dasgupta *et al.* 2008,

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Abbreviations: Chl − chlorophyll; Cyt b_{559} − cytochrome b_{559}; DEPMPO − 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide; DEPMPO-OOH − DEPMPO superoxide radical adduct; EPR − electron paramagnetic resonance; HP − high potential; HQ − hydroquinone; LP − low potential; MES − 2-(N-morpholino) ethanesulfonic acid; $O_2^{\cdot-}$ − superoxide radical; PSII − photosystem II; OEA − oxygen evolution activity; Q_A, Q_B − primary and secondary quinone electron acceptors of photosystem II; ROS − reactive oxygen species; SSE – $O_2^{\cdot-}$-scavenging efficiency; SOD − superoxide dismutase; TCNE − tetracyanoethylene; XO/X − xanthine oxidase/hypoxanthine.
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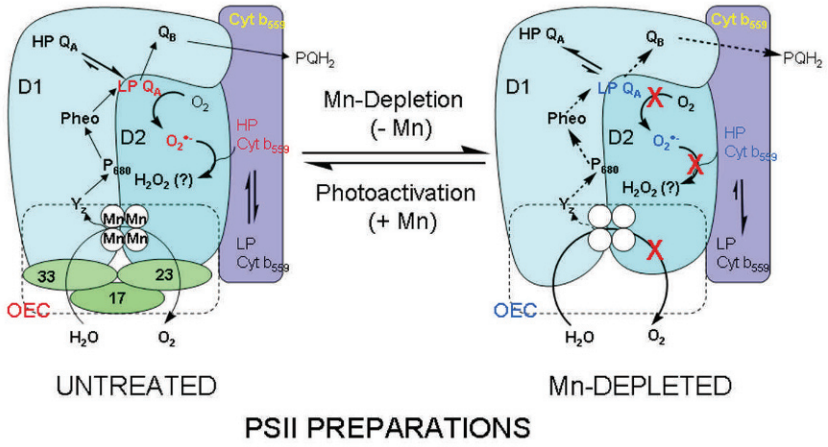

Fig. 1. Molecular pathway for the generation of $O_2^{\bullet-}$ in untreated PSII (*left*) from the active oxygen evolution complex (OEC) and simultaneous electron transfer from Mn cluster to PQH_2. Higher low potential (LP) Q_A and high potential (HP) Cyt b_{559} ratios indicate more $O_2^{\bullet-}$ generation and scavenging, respectively. Comparatively, in Mn-depleted PSII (right), the OEC is inactive resulting in low oxygen evolution activity and inefficient electron transfer with lower LP Q_A and HP Cyt b_{559} ratios, thereby leading to decreased $O_2^{\bullet-}$ production and removal.

Iwasaki *et al.* 1995). Following the repair process, the oxygen-evolving machinery is reassembled, and the corresponding functions are partially restored (Karacan and Somer 2004). If the repairing process of the damaged PSII is slower than the photodamage to PSII, the photoinhibition occurs (Nishiyama *et al.* 2006). Thus, the photoinhibition/repair cycle is crucial to maintain the activity and function of PSII, especially under photo-inhibited conditions.

The PSII photodamage induced by reactive oxygen species (ROS) has gained wide attention (Song *et al.* 2006, Tjus *et al.* 2001, Knox and Dodge 1985) and is proposed as an important photoinhibition mechanism. Interestingly, recent studies showed that ROS do not directly damage the PSII but inhibit the repair of the photodamaged PSII (Nishiyama *et al.* 2006 and reference therein). The generation of superoxide radical ($O_2^{\bullet-}$), one of the most important ROS, in untreated PSII preparations has been determined by various methods (Navari-Izzo *et al.* 1999, Cleland *et al.* 1999, Chen *et al.* 1992, *etc.*). Moreover, the *in vitro* Mn-depletion treatment of PSII also resulted in the $O_2^{\bullet-}$ production (Ananyev *et al.* 1994, Zhang *et al.* 2003). However, the $O_2^{\bullet-}$ production remains unknown in the photoactivation process of PSII. In fact, the Mn-depleted and subsequently photoactivated treatments of PSII can be considered as the *in vitro* photoinhibition/repair process. The study on the $O_2^{\bullet-}$ generation and scavenging in the Mn-depleted and photoactivated processes could shed light on the regulation of the $O_2^{\bullet-}$ levels by PSII *in vivo*.

As mentioned above, $O_2^{\bullet-}$ can be generated in various PSII preparations, while no superoxide dismutase (SOD) has been observed (Klimov *et al.* 1993, Ananyev *et al.* 1994, Kruk and Strzałka 1999, Nugent 2001). However, intrinsic SOD-like activities were detected in PSII (Klimov *et al.* 1993, Ananyev *et al.* 1994, Kruk and Strzałka 1999, Nugent 2001). For example, the 33 kDa protein together with part of manganese was firstly suggested as an intrinsic SOD activity (Ananyev *et al.* 1994, Zhang *et al.* 2003). In addition, various potential forms of cytochrome b_{559} (Cyt b_{559}) were important sources of the intrinsic SOD-like activity in the untreated PSII (Ananyev *et al.* 1994, Tiwari and Pospíšil 2009). Given that the Mn-depleted treatment of PSII loses the 33 kDa protein, Cyt b_{559} may be the main source of SOD activity in the Mn-depleted and photoactivated PSII. However, more recent study reported that low potential form of Cyt b_{559} took part in the production of $O_2^{\bullet-}$ (Pospíšil *et al.* 2006), leading to the complication of the Cyt b_{559} functions. Therefore, the SOD activity of Cyt b_{559} needs to be further revisited using new detection techniques.

In the present work, we firstly investigated the $O_2^{\bullet-}$ production in the untreated, Mn-depleted, or photo-activated PSII preparations using the spin trapping-EPR technique. Then, the role of Cyt b_{559} as a main SOD activity in PSII preparations was determined using the same technique. Furthermore, the variations of this intrinsic SOD activity and the $O_2^{\bullet-}$ levels in the Mn-depleted and photoactivation processes were investigated. The original $O_2^{\bullet-}$ levels upon depletion of the SOD activity were for the first time estimated in these two processes. Based on the close relationship between the intrinsic SOD activity and the $O_2^{\bullet-}$ level, a new photoprotective mechanism regarding biphasic regulation of the $O_2^{\bullet-}$ level by PSII itself was proposed.

Materials and methods

Preparation of PSII samples: PSII from normally grown spinach were isolated as previously described (Berthold *et al.* 1981) with some modifications (Yruela *et al.* 1991). Samples were resuspended in SMN buffer composed of 0.4 M sucrose, 50 mM MES-NaOH (pH 6.0) and 10 mM NaCl. For storage purposes, samples were frozen in liquid nitrogen for future use. Chlorophyll (Chl) concentration was determined according to the procedure described by Arnon (1949).

TCNE treatment: TCNE (0.2 mM) was added to the untreated PSII [0.25 mg(Chl) mL^{-1}]. The mixture was incubated for 5 min on ice in the absence of light with gentle stirring and then centrifuged at 27,000 × *g* for 10 min at 4ºC. The resulting pellet was washed three times with the SMN buffer and resuspended using the same buffer at a final concentration of 0.25 mg(Chl) mL^{-1} (Cleland and Grace 1999).

Mn-depleted treatment: In the absence of light, PSII [0.5 mg(Chl) mL^{-1}] in the medium containing 0.4 M sucrose, 10 mM $CaCl_2$ and 180 mM NaCl and 50 mM MES-NaOH (pH 6.0) was incubated with 5 mM NH_2OH at 4ºC for 30 min to remove Mn. Then, the Mn-depleted PSII was collected after centrifugation at 40,000 × *g* for 20 min and washed three times with the same medium used for the NH_2OH treatment (Chen *et al.* 1992).

Hydroquinone (HQ)-treatment of Mn-depleted PSII: The HQ solution (8 mM) was added to the Mn-depleted PSII [0.25 mg(Chl) mL^{-1}] on ice in the absence of light with gentle stirring for 30 min (Gadjieva *et al.* 1999). Then, the resulting samples were centrifuged at 27,000 × *g* for 10 min, washed three times with the SMN buffer and resuspended in the same medium at a final concentration of 0.25 mg(Chl) mL^{-1}.

Photoactivation of Mn-depleted PSII: Photoactivation of Mn-depleted PSII was performed according to the previously reported methods (Chen *et al.* 1995, Miller and Brudvig 1989) with minor revisions. In brief, various concentrations of $MnCl_2$ (0–0.4 mM) were added to the Mn-depleted PSII preparations in SMN buffer [0.25 mg(Chl) mL^{-1}, pH 6.0] supplemented with $CaCl_2$ (50 mM) and 2,6-dichorophenolindophenol (10 μM). The mixture was incubated at 25ºC for 10 min in the dark and then illuminated for 30 min with incandescent bulbs (the intensity of the white light at 5 μmol m^{-2} s^{-1}) with gentle stirring. The photoactivated PSII preparations were firstly washed with high-salt Ca^{2+} buffer (200 mM NaCl, 15 mM $CaCl_2$, 25 mM MES (pH 6.5) and 30 % ethylene glycol) supplemented with 1 mM ethylenediaminetetraacetic acid and then with high-salt Ca^{2+} buffer alone to remove the extra Mn ions.

Oxygen evolution activity: Steady-state oxygen evolution was measured with a Clark-type electrode thermostated at 20ºC (Yruela *et al.* 1991). The assay sample contained 20 μg(Chl) mL^{-1} PSII preparations, 5 mM $CaCl_2$, 10 mM NaCl, 25 mM MES (pH 6.0), 0.5 mM 2,5-dichloro-1,4-benzoquinone and 2 mM potassium ferricyanide. The untreated PSII preparations were found to liberate oxygen at a rate of 450–550 μmol(O_2) mg^{-1}(Chl) h^{-1} under continuous white light irradiation with photon flux density of 1,000 μmol m^{-2} s^{-1}.

EPR-spin trapping: To quantify the $O_2^{\bullet-}$ production in various PSII preparations, a spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide(DEPMPO, 10 mM) which was synthesized according to the previous procedure (Frejaville *et al.* 1995) was added to the PSII preparations [0.15 mg(Chl) mL^{-1}] and illuminated for 1 min by a continuous He-Ne laser (25 mW, 632.8 nm). EPR spectra were recorded on a *Bruker ESP 300* spectrometer (Germany) operating at X-band at room temperature. The relative $O_2^{\bullet-}$ yield was represented by the peak intensity of the low-field fourth EPR line of DEPMPO-OOH. The instrument settings were as follows: modulation amplitude 0.144 mT; receiver gain 3.2 × 10^5; modulation frequency 100 kHz; microwave power 12.8 mW; sweep width 15 mT; time constant 0.328 s, and sweep time 83.89 s.

To investigate the intrinsic SOD-like activity, various PSII preparations [0.3 mg(Chl) mL^{-1}] were incubated with hypoxanthine (0.2 mM) and xanthine oxidase (0.04 U mL^{-1}) (X/XO system) in the presence of 10 mM DEPMPO and 0.2 mM diethylenetriaminepentaacetic acid (DETAPAC) in the dark. The $O_2^{\bullet-}$-scavenging efficiency (SSE) of the PSII preparations was calculated using the following equation: SSE = $(H_0 - H_x)/H_0$ × 100%, where H_x and H_0 are the EPR signal intensities of the samples with and without the PSII preparations, respectively.

Quantification of various forms of Cyt b_{559} by optical method: Optical experiments were carried out on a *Shimadzu UV-3000* spectrophotometer (*Shimadzu Co*., Kyoto, Japan). Different thermodynamic forms of Cyt b_{559} in various PSII preparations were identified at 559 nm (Rivas *et al.* 1995) from the difference in the absorption spectrum (*i.e.*, reduced minus oxidized forms, respectively). The total amount of Cyt b_{559} (100%) was obtained from the dithionite-reduced minus ferricyanide-oxidized spectrum. The amount of HP Cyt b_{559} was estimated from the HQ-reduced minus ferricyanide-oxidized spectrum. The amount of low potential form of Cyt b_{559} (LP Cyt b_{559}) was the difference between the total amount of Cyt b_{559} and the amount of HP Cyt b_{559}. The Chl concentration of PSII preparations in the measurements was 75 μg(Chl) mL^{-1} (Gadjieva *et al.* 1999).

Results

The $O_2^{\bullet-}$ production in various PSII preparations: Although the $O_2^{\bullet-}$ productions in the untreated PSII and Mn-depleted PSII were reported, it is unknown in the photoactivation process of Mn-depleted PSII. In order to obtain an insight into the $O_2^{\bullet-}$ production in the photoinhibition/repair cycle, the EPR-spin trapping technique using DEPMPO as a spin trap was utilized to compare the $O_2^{\bullet-}$ levels in various PSII preparations. As expected,

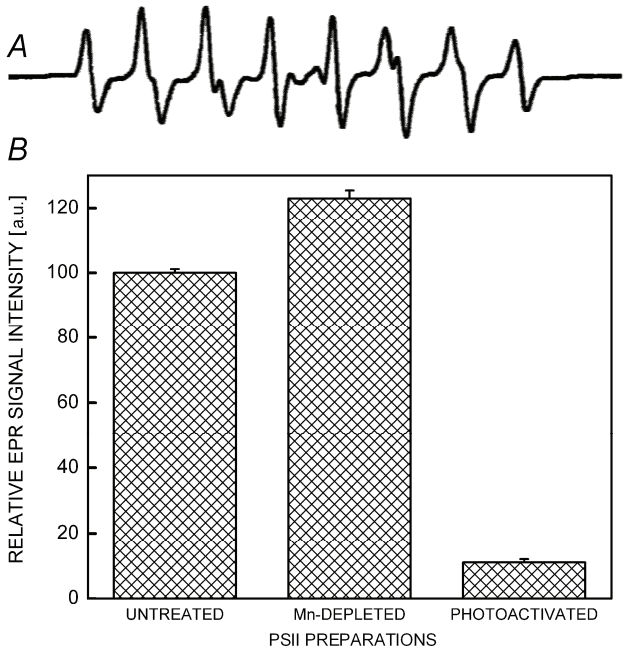

Fig. 2. (*A*) EPR spectrum of DEPMPO-OOH from illumination of photoactivated PSII [0.15 mg(Chl) mL^{-1}] in the presence of 10 mM DEPMPO; (*B*) relative EPR signal intensity of DEPMPO-OOH in the untreated PSII, Mn-depleted PSII and photoactivated PSII preparations. Each data represents mean value of at least three measurements ± SD. **p*<0.05, untreated PSII versus Mn-depleted PSII.

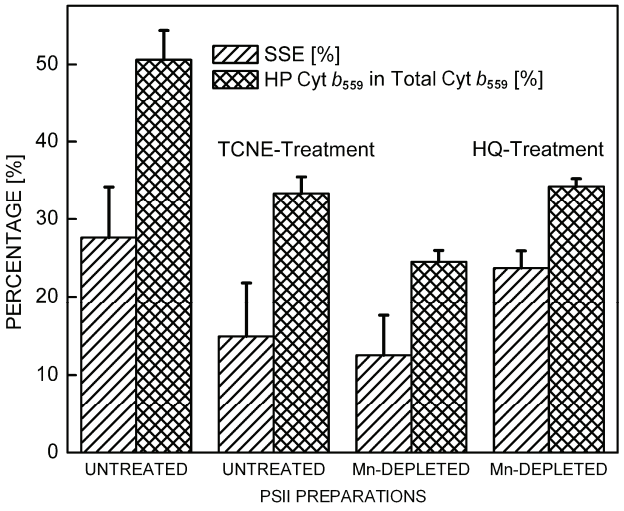

Fig. 3. Effect of TCNE- and HQ-treatments on the $O_2^{\bullet-}$-scavenging efficiency (SSE) and HP Cyt b_{559} percentage in the untreated PSII and Mn-depleted PSII preparations. EPR spectra were recorded 10 min after 0.04 U mL^{-1} xanthine oxidase was added into the solution containing 0.2 mM hypoxanthine, 0.2 mM DETAPAC and 0.3 mg(Chl) mL^{-1} PSII preparations in the SMN buffer (pH 6.0). Each data represents mean value of at least three measurements ± SD.

EPR signals of DEPMPO superoxide spin adduct (DEPMPO-OOH) were consistently observed when illuminating various PSII preparations. [Note: although there is an equilibrium between $O_2^{\bullet-}$ and its protonated form ($HOO^{\bullet}$), both species which afford the same spin adduct (DEPMPO-OOH) after spin trapping by DEPMPO will not be distinguished throughout the paper. Therefore, these two species are consistently called as "superoxide radical" and its corresponding abbreviation ($O_2^{\bullet-}$) is applied]. Fig. 2*A* showed the typical EPR signal of DEPMPO-OOH from the photoactivated PSII. No EPR signal was observed from the above experiments before illumination (data not shown), indicating that the $O_2^{\bullet-}$ generation is light-driven. Correct assignment of the DEPMPO-OOH signal was based on the following facts: (*1*) its EPR spectral profile is completely identical with that in the X/XO $O_2^{\bullet-}$ generating system (Frejaville *et al.* 1995); (*2*) the signals were completely eliminated by addition of exogenous SOD (data not shown). Fig. 2*B* showed the relative EPR signal intensities of DEPMPO-OOH observed in various preparations. The Mn-depleted treatment of PSII resulted in the slight increase of the $O_2^{\bullet-}$ level (123%) compared to that in the untreated PSII (100%) while the $O_2^{\bullet-}$ level was greatly reduced (11% relative to that in the untreated PSII preparations) upon photoactivation of the Mn-depleted PSII using 0.05 mM $MnCl_2$. The $O_2^{\bullet-}$ level measured in the above systems is net because the intrinsic SOD analogue may quench a part of $O_2^{\bullet-}$. In order to gain insight into the original $O_2^{\bullet-}$ level (the level measured under the complete inhibition of the intrinsic SOD activity) and the regulation of the $O_2^{\bullet-}$ level by PSII, the intrinsic SOD activity is determined in PSII preparations.

HP Cyt b_{559} as a main endogeneous SOD analogue in the untreated PSII: HP Cyt b_{559} was previously suggested as an endogeneous SOD analogue in the untreated PSII preparations using ferricytochrome *c* reduction and xanthine/oxidase assays (Ananyev *et al.* 1994). However, the source of the intrinsic SOD activity as well as the roles of Cyt b_{559} in active PSII still remains unclear. In order to verify the presence of the endogenous SOD activity in PSII, we investigated the $O_2^{\bullet-}$-scavenging ability of the PSII preparations in the X/XO $O_2^{\bullet-}$ generating system using the EPR-spin trapping technique. Moreover, the effect of TCNE and HQ on the $O_2^{\bullet-}$-scavenging efficiency (SSE) was also investigated in the untreated PSII and Mn-depleted PSII preparations. While TCNE can transform HP Cyt b_{559} into LP Cyt b_{559} in untreated PSII (Ananyev *et al.* 1994), HQ recovers HP Cyt b_{559} from LP Cyt b_{559} in the Mn-depleted PSII (Gadjieva *et al.* 1999). The relative amount of LP Cyt b_{559} and HP Cyt b_{559} in various PSII preparations was quantified using optical method. As shown in Fig. 3, the percentage of HP Cyt b_{559} in the total Cyt b_{559} was approximately 50% in the untreated PSII, consistent with the reported value (Gadjieva *et al.* 1999). Correspondingly, the $O_2^{\bullet-}$-scavenging efficiency (SSE) in the untreated PSII was 28% in the dark. Treatment of the

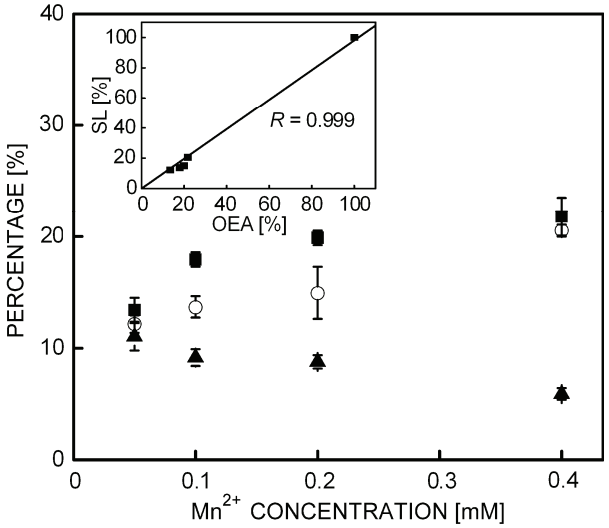

Fig. 4. The oxygen evolution activity (OEA) (■) as well as the $O_2^{\bullet-}$ levels in the presence (○) and absence (▲) of the instrinsic SOD activity in the Mn-depleted PSII after the photoactivated treatment with 0–0.4 mM $MnCl_2$. The *inset* shows the linear relationship between OEA and the original $O_2^{\bullet-}$ level (SL) measured by depletion of the instrinsic SOD activity in the reconstituted Mn-depleted PSII preparations. Each data represents the mean value of at least three measurements ± SD.

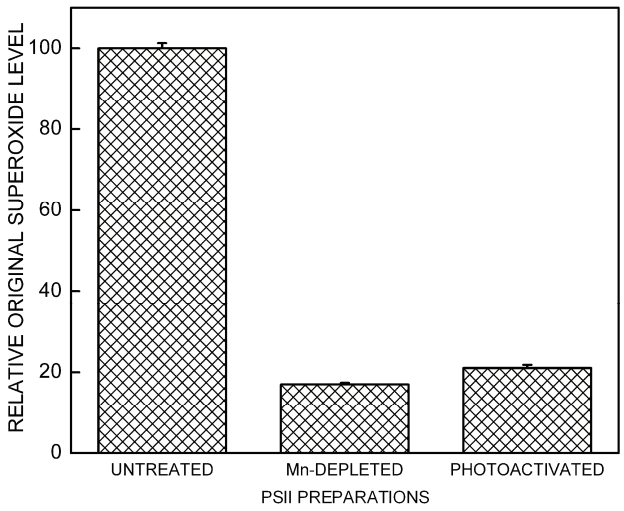

Fig. 5. The relative original $O_2^{\bullet-}$ levels in the untreated, Mn-depleted and photoactived PSII preparations upon inhibiting the intrinsic SOD activity by using TCNE. The photoactivated treatment was carried out using 0.4 mM $MnCl_2$. Each data represents the mean value of at least three measurements ± SD. *p<0.01, Mn-depleted PSII *vs*. photoactivated PSII.

PSII preparations with TCNE resulted in the partial transformation of HP Cyt b_{559} into LP Cyt b_{559} with a decreased HP Cyt b_{559} ratio (33%) and SSE (15%). On the other hand, the Mn-depleted treatment of the untreated PSII preparations reduced the amount of HP Cyt b_{559} to 24% (Ananyev *et al.* 1994, Gadjieva *et al.* 1999) with 12% SSE. It is clear that both TCNE and Mn-depletion treatments inhibit the intrinsic SOD-like activity in PSII preparations. Of note is that subsequent incubation of the Mn-depleted PSII preparations with 8 mM HQ restored the intrinsic SOD-like activity up to 86% of the untreated PSII preparations with 24% SSE and 34% HP Cyt b_{559}. Highly efficient restoration of the SOD activity by HQ suggests that the intrinsic SOD-like activity in the PSII preparations mainly originates from HP Cyt $b_{559,}$ although the intermediate potential form of Cyt b_{559} has been also shown to contribute the $O_2^{\bullet-}$ scavenging in PSII (Tiwari and Pospíšil 2009).

Effect of the photoactivation treatment on the original and net $O_2^{\bullet-}$ levels in the Mn-depleted PSII: In the photoactivated experiment, $MnCl_2$ was used as an exogenous source of Mn ions to reconstitute the Mn cluster in the Mn-depleted PSII. In order to avoid the intervention from the 33 kDa protein which was suggested to exert an intrinsic SOD-like activity (Ananyev *et al.* 1994, Zhang *et al.* 2003), no exogenous 33 kDa protein was introduced in this experiment. Since the oxygen evolution activity (OEA) is an important parameter to identify the photoactivation efficiency of the Mn-depleted PSII preparations, it was measured *in situ* by the Clark electrode. As shown in Fig. 4, OEA from the Mn-depleted PSII preparations was restored upon the photoactivation treatments and its efficiency strongly depends on the $MnCl_2$ concentration. Initially, OEA of the Mn-depleted PSII alone was negligible. After incubation with 0.4 mM of $MnCl_2$, OEA from the Mn-reconstituted preparations restored to 23% of the value from the untreated PSII preparation (Fig. 4). Attempt to increase the $MnCl_2$ concentrations did not lead to higher OEA (data not shown). As reported previously, the relatively low OEA in the photoactivation process using $MnCl_2$ could be improved by using the synthesized Mn complexes (Liu *et al*. 2006, Allakhverdiev *et al*. 1999, 1994).

In order to reveal the variations in the $O_2^{\bullet-}$ production and scavenging during photoactivation of the Mn-depleted PSII, the relative $O_2^{\bullet-}$ levels were measured in the presence and absence of TCNE. As shown in Fig. 4, the $O_2^{\bullet-}$ levels in the Mn-reconstituted PSII decreased with the $MnCl_2$ concentrations in the absence of TCNE. In contrast, in the presence of TCNE, the levels increased upon the photoactivation. At the low concentration of $MnCl_2$ (0.05 mM), TCNE did not have a significant effect on the $O_2^{\bullet-}$ levels (approximately 12% of the untreated PSII) in the reconstituted PSII preparations (Fig. 4). However, at the higher concentration of $MnCl_2$, the effect of TCNE is more pronounced. For example, when the $MnCl_2$ concentration increased to 0.4 mM, the $O_2^{\bullet-}$ level was increased to 22% in the presence of TCNE compared to 6% in the absence of TCNE. Given that HP Cyt b_{559} contributes to the main SOD-like activity in the PSII, the original $O_2^{\bullet-}$ levels in the three PSII preparations were measured using the EPR-spin trapping technique in the presence of TCNE. Indeed, measurement of the original

$O_2^{\bullet-}$ levels in various PSII preparations is essential to investigate the effect of the PSII itself on the $O_2^{\bullet-}$ levels (*i.e.*, the regulation of the $O_2^{\bullet-}$ levels by PSII itself) in the *in vitro* photoinhibition/repair process. As shown in Fig. 5, the Mn-depleted treatment greatly reduced the original $O_2^{\bullet-}$ level (17%) as comparison to the level in the untreated PSII (100%) but the subsequent photoactivated treatment of the Mn-depleted PSII with 0.4 mM $MnCl_2$ effectively restored the original $O_2^{\bullet-}$ level to 21%. Interestingly, the original $O_2^{\bullet-}$ levels were well corrected with the OEA (Fig. 4, *inset*), implying that the $O_2^{\bullet-}$ generation depends on the electron transfer chain in PSII.

Discussion

In this study, our overall goal is to determine if PSII itself can effectively regulate the $O_2^{\bullet-}$ level upon the Mn-depleted and photoactivated treatment. In doing so, we firstly detected the $O_2^{\bullet-}$ levels in various PSII preparations. As shown in Fig. 2, illumination of both untreated and Mn-depleted PSII preparations led to the $O_2^{\bullet-}$ production as reported previously (*e.g*. Ananyev *et al.* 1994, Zhang *et al.* 2003). In addition, the $O_2^{\bullet-}$ production was for the first time observed in the photoactivated PSII. Interestingly, the $O_2^{\bullet-}$ levels in these PSII preparations were different as evidenced by the strongest EPR signal of DEPMPO-OOH in the Mn-depleted PSII and lowest in the photoactivated PSII.

Indeed, the $O_2^{\bullet-}$ levels measured in the experiments rely on the $O_2^{\bullet-}$ generation and scavenging. Although no CuZn-SOD or Mn-SOD were reported in PSII, the intrinsic SOD activity has been proposed in PSII which includes extrinsic 33 kDa polypeptide together with part of Mn as well as various forms of Cyt b_{559}. Recent studies showed that the intermediate potential (IP) form of Cyt b_{559} serves as $O_2^{\bullet-}$ oxidase that catalyzes the one-electron oxidation of $O_2^{\bullet-}$ to O_2, and the reduced high potential form of Cyt b_{559} acts as $O_2^{\bullet-}$ reductase known to catalyze the one-electron reduction of $O_2^{\bullet-}$ to H_2O_2. In the present study, our result from the $O_2^{\bullet-}$-scavenging experiments by the PSII preparations verified the presence of the SOD activity in PSII (Fig. 3). Using TCNE and HQ to modulate the level of HP Cyt b_{559}, we further found that the intrinsic SOD activity has a very close relationship with the HP Cyt b_{559} level in the PSII preparations. High restoration (86%) of the SOD activity by HQ treatment of the Mn-depleted PSII implies that HP Cyt b_{559} exerts a main SOD activity in PSII. The SOD activity of HP Cyt b_{559} can be reasonably explained by its similarities in structural and redox potential properties with various kinds of SODs, such as CuZn-SOD and Mn-SOD (Ananyev *et al.* 1994). Therefore, the role of residual Mn together with the extrinsic 33 kDa polypeptide as a SOD activity in PSII is negligible in our experiments although the Mn-depleted treatment could not completely remove them (Miller and Brudvig 1989).

Upon inhibition of the intrinsic SOD activity by TCNE, the original $O_2^{\bullet-}$ levels were quantified in various PSII preparations. As shown in Fig. 5, the original $O_2^{\bullet-}$ level in the Mn-depleted PSII is much lower than that in the untreated PSII. While the photoacitvated treatment restores the OEA, it also increases the original $O_2^{\bullet-}$ production.

Good linearity between the original $O_2^{\bullet-}$ level and the OEA in the photoactivated PSII verifies that the $O_2^{\bullet-}$ production in PSII has a close relationship with the electron transfer chain (Fig. 4, *inset*). The plastoquinone molecules Q_A and Q_B, as the primary and secondary quinone electron acceptors in PSII, respectively, have been suggested to be the most possible reductants of molecular oxygen which are responsible for the $O_2^{\bullet-}$ generation (Ananyev *et al.* 1994, Cleland and Grace 1999, Pospíšil *et al.* 2004, Liu *et al.* 2004). In fact, the Mn-depletion treatment changes the redox state of Q_A from low potential form (E_m = –80 mV) to the corresponding high potential (E_m = +110 mV) (Johnson *et al*. 1995) and thus interrupt the forward electron transfer to Q_B (Krieger-Liszkay 2005, Johnson *et al.* 1995, Krieger *et al.* 1995) or molecular oxygen. This process directly inhibits the $O_2^{\bullet-}$ generation from Q_A or indirectly from Q_B. Therefore, the original $O_2^{\bullet-}$ level in the Mn-depleted PSII is relatively lower than that in untreated PSII because of the interruption of the electron transfer chain as well as the variation of the redox potential of Q_A. Upon the photoactivation, the OEA and electron transfer activity in the Mn-depleted PSII were partially restored (Fig 4). The midpoint redox potential of Q_A is accordingly converted from high potential back to low potential (Johnson *et al*. 1995) and therefore the original $O_2^{\bullet-}$ level increases (Fig. 4 and Fig. 5).

On the other hand, the Mn-depleted treatment led to the conversion of the midpoint redox potential of Cyt b_{559} from +435 mV to +45 mV (Mizusawa *et al.* 1995, Iwasaki *et al*. 1995), thus inhibiting its SOD activity. As a result, $O_2^{\bullet-}$ can be still detected in the Mn-depleted treatment although the original $O_2^{\bullet-}$ level is decreased. During the photoactivation, the restoration of the electron transfer activity causes the conversion of LP Cyt b_{559} into HP Cyt b_{559} and this process has been suggested as the primary one in photoreaction (Mizusawa *et al*. 1997, 1999). Therefore, the SOD activity exerted by HP Cyt b_{559} effectively decreases the net $O_2^{\bullet-}$ level produced in the photoactivation process (Figs. 2, 4).

Our above results suggest that PSII can modulate the net $O_2^{\bullet-}$ level through simultaneous regulation of the midpoint redox potentials of Cyt b_{559} and Q_A, thereby keeping the $O_2^{\bullet-}$ level under control. Photoactivation increases the original $O_2^{\bullet-}$ level with the restoration of the electron transfer activity. In order to "smoothen" the normal 'repair cycle' and to avoid the probable photooxidation induced by excess of $O_2^{\bullet-}$ and $O_2^{\bullet-}$-

derived ROS, the intrinsic SOD-like activity of PSII from HP Cyt b_{559} is primarily restored and results in the decrease of the net $O_2^{\bullet-}$ level (Figs. 1, 2, 4). On the contrary, the Mn-depletion decreases the original $O_2^{\bullet-}$ level due to the inactivation of Q_A as well as lack of electron donors. The moderately residual $O_2^{\bullet-}$ level allows for the maintenance of the physiological roles of $O_2^{\bullet-}$ and $O_2^{\bullet-}$-derived ROS (Figs. 1, 2, 5).

Over the past decades, it has been clearer that ROS including $O_2^{\bullet-}$ play an important role in the pathophysiology of plants. For example, ROS like H_2O_2 act as a key signaling molecule for growth and development, second programmed metabolisms, biotic stress responses (Bhattacharjee *et al.* 2005) and are involved in different signaling pathways for defense mechanisms, such as triggering of the hypersensitive response, accumulation of phytoalexins and a number of other defense-response genes (Guan and Scandalios 2000, Levine *et al.* 1994). On the contrary, excess of ROS causes oxidative damage to proteins, DNA, and lipids (Apel and Hirt 2004), destroys PSII oxygen evolution activity (Song *et al.* 2006) and blocks the repair process of PSII (Nishiyama *et al.* 2006). Therefore, the regulation of the ROS levels is of significant importance for many metabolic processes in plant.

In conclusion, the original $O_2^{\bullet-}$ level in the Mn-depleted and photoactivated PSII were for the first time detected using the EPR-spin trapping technique. A new mechanism regarding biphasic regulation of the $O_2^{\bullet-}$ levels by PSII *via* simultaneously regulating the midpoint redox potentials of Cyt b_{559} and Q_A was proposed. The present study should open up a new possibility regarding the "self-healing" role of PSII in the photoinhibition/ repair cycle.

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