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Review

A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II

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Abstract

Inhibition of the activity of photosystem II (PSII) under strong light is referred to as photoinhibition. This phenomenon is due to the imbalance between the rate of photodamage to PSII and the rate of the repair of damaged PSII. Photodamage is initiated by the direct effects of light on the oxygen-evolving complex and, thus, photodamage to PSII is unavoidable. Studies of the effects of oxidative stress on photodamage and subsequent repair have revealed that reactive oxygen species (ROS) act primarily by inhibiting the repair of photodamaged PSII and not by damaging PSII directly. Thus, strong light has two distinct effects on PSII; it damages PSII directly and it inhibits the repair of PSII via production of ROS. Investigations of the ROS-induced inhibition of repair have demonstrated that ROS suppress the synthesis de novo of proteins and, in particular, of the D1 protein, that are required for the repair of PSII. Moreover, a primary target for inhibition by ROS appears to be the elongation step of translation. Inhibition of the repair of PSII by ROS is accelerated by the deceleration of the Calvin cycle that occurs when the availability of CO_2 is limited. In this review, we present a new paradigm for the action of ROS in photoinhibition.

Keywords: Photoinhibition; Photosystem II; Protein synthesis; Reactive oxygen species; Repair

1. Introduction

Light is a prerequisite for photosynthesis but it is harmful to the photosynthetic machinery. Exposure of photosynthetic organisms to strong light often results in severe inhibition of the activity of photosystem II (PSII) [1–4]. This phenomenon is referred to as the photoinhibition of PSII. The light-dependent damage (photodamage) to PSII occurs under light of any intensity and is, thus, unavoidable in photosynthetic organisms [5–7]. Photosynthetic organisms are able, however, to overcome photodamage by the rapid and efficient repair of PSII under normal light conditions [2–4]. This repair requires protein synthesis. The rate of photodamage is proportional to the intensity of light [6–9], whereas the rate of repair is highest at a certain intensity of light [10]. When photosynthetic cells are exposed to weak light, the rate of repair is higher than the rate of photodamage and, thus, photoinhibition does not occur apparently. However, when cells are exposed to strong light, the rate of photodamage exceeds the rate of repair, resulting in the photoinhibition of PSII.

Light can also generate oxidative stress by producing reactive oxygen species (ROS) as inevitable by-products of photosynthesis (Fig. 1). Reduction of oxygen on the acceptor side of photosystem I (PSI), as a result of the photosynthetic transport of electrons, leads to the formation of the superoxide radical (O_2^-), which can be further converted to hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$) [11]. Transfer of excitation energy from excited chlorophylls to oxygen in the light-harvesting complexes leads to the formation of singlet-state oxygen (singlet oxygen; $^1O_2$) [12,13]. The levels of these various ROS can be reduced to tolerable levels by antioxidative systems that include ROS-scavenging enzymes, such as superoxide dismutase and ascorbate peroxidase, as well as antioxidants, such as β-carotene

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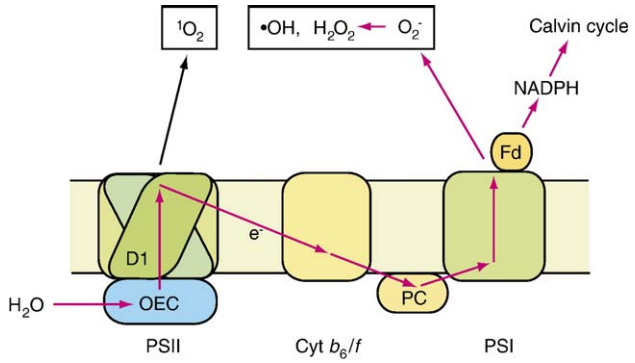

Fig. 1. The production of ROS in the photosynthetic machinery on the thylakoid membrane. Reduction of oxygen on the acceptor side of PSI, as a result of the photosynthetic transport of electrons, leads to the formation of O_2^-, which can be further converted to H_2O_2 and •OH. Transfer of excitation energy from excited chlorophylls to oxygen in the light-harvesting complexes leads to the formation of $^1O_2$. $^1O_2$ can also be produced from non-functional PSII in which the oxygen-evolving complex has been damaged. The production of ROS is enhanced by strong light and also by deceleration of the Calvin cycle. Red arrows indicate the transport of electrons and the black arrow indicates the transfer of excitation energy. Fd, Ferredoxin; OEC, the oxygen-evolving complex; PC, plastocyanin.

and α-tocopherol, under non-stress conditions [11,14]. However, when the absorption of strong light exceeds the capacity of the photosynthetic machinery for photosynthesis, the production of ROS is accelerated and elevated levels of ROS give rise to oxidative stress [11].

Direct involvement of ROS in the photodamage to PSII has been a subject of controversy. Some investigations suggested that ROS and, in particular, $^1O_2$ are the primary cause of photodamage to PSII. These investigations led to the two proposed mechanisms, namely, the "acceptor-side" mechanism [15] and "charge-recombination" mechanism [16]. Generation of $^1O_2$ can be detected in isolated PSII complexes, in isolated thylakoid membranes, and in plant leaves during illumination [17–20]. Exposure of isolated thylakoid membranes to exogenous $^1O_2$ results in specific cleavage of the D1 protein, an essential component of PSII [21]. Other types of ROS, such as O_2^-, H_2O_2, and •OH, also induce the specific cleavage of the D1 protein in vitro [22–25]. However, mechanisms of photodamage to PSII that are not mediated by ROS have also been proposed. In the proposed "donor-side" mechanism, acidification of the lumen, due to the transfer of protons across the thylakoid membrane, inactivates the oxygen-evolving system and allows $P680^+$, the oxidized reaction-center chlorophyll, to survive for an extended period of time, and this strong oxidant damages the D1 protein [24,26–28].

Evidence for these putative mechanisms has been drawn from studies in vitro of isolated PSII complexes and isolated thylakoid membranes. Thus, it remains unclear whether these mechanisms are actually involved in the photoinhibition of PSII in living cells. In addition, materials examined in vitro have lost the ability to repair PSII and, thus, the effects of ROS on repair cannot be investigated in such systems.

Methods for the separate monitoring of photodamage to and repair of PSII in vivo have been established in cyanobacteria and plants [5,29,30]. Their application has revealed that ROS act primarily by inhibiting the repair of PSII and not by damaging PSII directly [9,10,31,32] and that photodamage to PSII is a light-dependent event, which is not initiated by ROS [33,34]. In this review, we summarize recent progress in studies of photoinhibition and propose a new paradigm for the action of ROS in the photoinhibition of PSII.

2. ROS do not accelerate photodamage to PSII but inhibit the repair of PSII

In living cells, photodamaged PSII is repaired by a rapid and efficient repair system [2–4]. The activity of PSII under given light conditions is influenced by both damage and repair processes. Thus, in order to monitor the process of damage exclusively, it is necessary to block the repair process by incubating cells in the presence of an inhibitor of protein synthesis, such as chloramphenicol or lincomycin. The repair of PSII can be observed after exposure of cells to very strong light, which reduces the activity of PSII to 10 to 20% of its initial level, and subsequent exposure of cells to weak light [5,29,30].

The effects of ROS on the photodamage to and repair of PSII were examined separately in cells of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) [9,31]. Increases in intracellular levels of H_2O_2, as a result of the inclusion of 0.5 mM H_2O_2 in the culture medium or inactivation of genes for H_2O_2-scavenging enzymes, stimulated the apparent photoinhibition of PSII by inhibiting the repair of PSII but not by accelerating photodamage to PSII [31]. Increases in intracellular levels of $^1O_2$, by illumination of cells in the presence of either 20 μM rose bengal or 20 μM ethyl eosin, also stimulated the apparent photoinhibition of PSII by inhibiting the repair of PSII but not by accelerating photodamage to PSII [9]. These observations indicated that ROS act primarily by inhibiting the repair of PSII under oxidative conditions.

3. Protein synthesis is a specific target of the actions of ROS

Photodamaged PSII is repaired in several steps, as follows: proteolytic degradation of the D1 protein; synthesis de novo of the precursor to D1 (known as pre-D1); insertion of the newly synthesized pre-D1 into the thylakoid membrane concomitantly with the assembly of other components of PSII; maturation of the D1 protein via the carboxy-terminal processing of pre-D1; and the assembly of the oxygen-evolving machinery [2,3,35–39]. Thus, repair of PSII can be traced by monitoring the turnover of the D1 protein.

The synthesis of the D1 protein de novo was markedly suppressed in *Synechocystis* cells in which the repair of PSII had been inhibited by elevated intracellular levels of H_2O_2 and $^1O_2$ [9,10,31]. The cited studies also revealed that H_2O_2 and $^1O_2$ suppressed not only the synthesis of the D1 protein but also the synthesis of almost all the proteins in thylakoid membranes, suggesting that the target of suppression of repair might be a process common to the synthesis of all proteins. By contrast, degradation of the D1 protein was unaffected by increases in intracellular levels of H_2O_2 and $^1O_2$ [9,31]. This observation is consistent with the finding that the presence or generation of

H_2O_2 or $^1O_2$ does not accelerate photodamage to PSII, as mentioned above.

An attempt was made to identify the site of inhibition of repair by these ROS in the sequence of steps that lead to the synthesis of the D1 protein de novo in *Synechocystis*, namely, transcription of the *psbA* gene for D1, translation of *psbA* mRNA, and processing of pre-D1. Northern and immunoblotting analyses revealed that the translation of *psbA* mRNA was specifically suppressed by H_2O_2 [31] and $^1O_2$ [9]. Subcellular localization of polysomes with bound *psbA* mRNA suggested that a primary target of H_2O_2 and $^1O_2$ might be the elongation step of translation [9,31]. Thus, it is likely that the suppression by ROS of the translational elongation of the product of *psbA* mRNA results in the inhibition of the repair of PSII during the photoinhibition of PSII (Fig. 2).

Translational elongation is known to be sensitive to oxidative stress [40]. In particular, elongation factor G is sensitive to oxidation by H_2O_2 in *Escherichia coli* [41,42], and this factor has also been identified as one of the proteins that are most susceptible to oxidation in a mutant of *Escherichia coli* that lacks a superoxide dismutase [43]. The eukaryotic counterpart of elongation factor G, elongation factor 2, is selectively inactivated by the oxidants cumene hydroperoxide and H_2O_2 [40,44]. Thus, elongation factors appear to be the most probable candidates for primary targets of H_2O_2 and $^1O_2$ in cyanobacteria and plants.

The light-dependent synthesis of pre-D1 is regulated at the translational level in plants [45,46] and algae [47] and at both the transcriptional and the translational level in cyanobacteria [48,49]. In *Chlamydomonas*, initiation of the translation of *psbA* mRNA was activated by the redox-active protein RB60, which was regulated by reducing equivalents from PSI, and also by a priming signal from the plastoquinone pool [47,50,51]. By contrast, in higher plants, elongation of the product of translation of *psbA* mRNA was regulated by redox signals that resulted from electron transport via PSI [52,53] or by a proton gradient across the thylakoid membrane, which was generated by the transport of electrons [54]. The synthesis of ATP was required for activation of the translation of *psbA* mRNA in *Synechocystis* [55] and in chloroplasts from spinach leaves [56]. Thus, a reducing signal from PSI and the synthesis of ATP might be expected to play an important role in the light-dependent activation of the translation of *psbA* mRNA. We can assume that, under oxidative conditions, ROS interrupt the normal redox signal(s) that would otherwise activate the translation of *psbA* mRNA. A recent biochemical study demonstrated that elongation factor 2 interacts with thioredoxin, suggesting that the reduction of disulfide bonds in elongation factors might be involved in the activation of translational elongation [57]. Excess ROS might interrupt such a reducing signal by oxidizing specific thiol-containing residues of elongation factors so that the translational elongation of *psbA* mRNA is arrested.

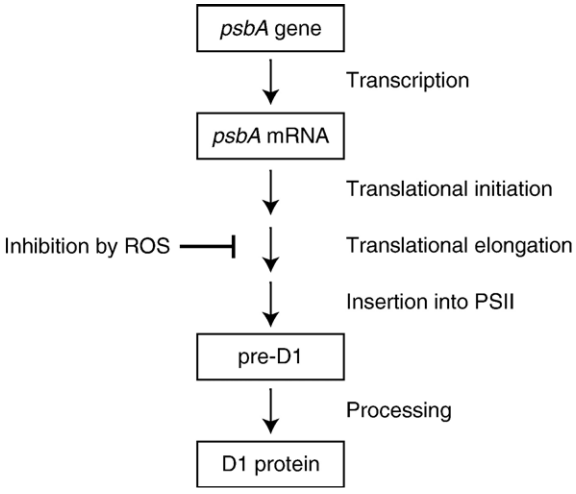

Fig. 2. A model for the action of ROS in the photoinhibition of PSII. Inhibition of the repair of PSII is attributable to suppression by ROS of the synthesis de novo of proteins and, in particular, of the D1 protein, that are required for the repair of PSII. The synthesis of the mature D1 protein involves a sequence of steps, namely, transcription of the *psbA* gene, translation of *psbA* mRNA, insertion of pre-D1 into PSII, and processing of pre-D1. Among these steps, it is the translational elongation of the product of *psbA* mRNA that appears to be particularly sensitive to ROS.

4. Evaluation of previously proposed mechanisms of photodamage to PSII

What does photodamage to PSII involve? Separation of photodamage from repair in vivo revealed many aspects of the nature of photodamage. For example, the initial rate of photodamage to PSII is exactly proportional to the intensity of the incident light [6–10,58]. This relationship cannot be fully explained by the "acceptor-side", "charge-recombination", or "donor-side" mechanism, none of which would generate a linear relationship between light intensity and photodamage.

Moreover, photodamage to PSII is independent of the transport of electrons. Photodamage was unaffected by interruption of electron transport via PSII by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [9,55] and by acceleration of electron transport via PSI by methyl viologen [34,55]. Again, these phenomena cannot be explained by the three proposed mechanisms, all of which should be influenced by the rate of electron transport.

Finally, photodamage to PSII is unaffected by elevated levels or the overproduction of $^1O_2$ and H_2O_2 in cells, as mentioned above, and by the elimination of oxygen from cells [9,10,31]. Thus, ROS that include $^1O_2$ are unlikely to be the primary cause of photodamage to PSII.

Oxygen molecules might be sheltered from the reaction-center chlorophyll P680 by a hydrophobic environment around the reaction center, which would prevent the formation of $^1O_2$, and oxygen molecules that are evolved at the manganese cluster might be directed outward by a specific oxygen channel [7,59–61]. Thus, $^1O_2$ might be generated in non-functional PSII, in which the putative oxygen channel has already been disrupted as a result of photodamage and then oxygen molecules can approach P680 and receive excitation energy from the triplet state of P680 [7,60].

5. A new model for the mechanism of photodamage to PSII

The recent reexamination of photodamage to PSII has resulted in a new proposed mechanism of photodamage, which is different from those proposed previously. Studies of the

effects of monochromatic light on photodamage have revealed that photodamage to PSII occurs in two steps, with primary damage by UV and strong blue light occurring at the oxygen-evolving complex and secondary damage by light absorbed by photosynthetic pigments occurring at the reaction center of PSII (Fig. 3; [33]). The action spectrum of photodamage to PSII is completely different from the absorption spectra of chlorophylls or carotenoids [33,34,62–64] but resembles those of model manganese compounds [34,65,66]. Release of manganese ions from the oxygen-evolving complex is accompanied by photodamage to PSII, suggesting that disruption of the manganese cluster upon perception of light might be a primary event in photodamage [34,67]. This mechanism can also account for the nature of photodamage to PSII as described above, namely, the proportionality between light intensity and photodamage and the independence of photodamage from electron transport and ROS.

Once the oxygen-evolving complex is damaged, the supply of electrons from water to $P680^+$ is blocked, and levels of $P680^+$ remain high. Since $P680^+$ is a strong oxidant [68], it damages the reaction center by oxidizing the surrounding amino acid residues of proteins and, in particular, of the D1 protein. Alternatively, the light-induced impairment of the oxygen-evolving complex might allow the free access of oxygen molecules to P680, yielding $^1O_2$ or other ROS that can also damage the reaction center oxidatively [7,60]. In either case, impairment of the functions of the oxygen-evolving complex is likely to be the rate-limiting step in the overall process of photodamage to PSII.

6. How does the fixation of CO_2 contribute to photoinhibition?

The Calvin cycle is adversely affected by various types of environmental stress, such as low-temperature stress and salt stress [69]. The susceptibility of PSII to photoinhibition is enhanced when the Calvin cycle is suppressed under such environmental stress [69]. However, the question of whether suppression of the Calvin cycle might affect photodamage to PSII or the repair of PSII was addressed only recently. Interruption of the Calvin cycle by exogenous glycolaldehyde, an inhibitor of phosphoribulokinase, or by a missense mutation in the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) did not accelerate photodamage to PSII but inhibited the repair of PSII [70]. The inhibition of repair was attributable to inhibition of the synthesis de novo of proteins and, in particular, of the D1 protein at the translational level [70]. It is likely that inhibition was caused by the generation of ROS as a result of the interruption of the fixation of CO_2 [70]. Exogenous glycerate-3-phosphate (3-PGA), a product of the Calvin cycle, abolished the inhibitory effects of glycolaldehyde on the synthesis of proteins de novo, suggesting that 3-PGA accepted electrons from NADPH and decreased the levels of ROS that inhibited the synthesis of proteins [71]. Thus, it seems likely that suppression of the Calvin cycle in light promotes the generation of ROS such that PSII becomes increasingly susceptible to photoinhibition as a result of the ROS-induced inhibition of repair.

7. Effects of salt stress on photoinhibition

Salt stress is an environmental factor that limits the growth and productivity of plants [72,73]. In natural environment, salt stress often occurs in combination with light stress. There have been several studies of the effects of salt stress on PSII under strong light. Such studies suggest that salt stress enhances the photoinhibition of PSII, for example, in higher plants [74,75], in *Chlamydomonas* [76], and in a cyanobacterium [77]. However, since the cited studies were performed under conditions that negatively impact the repair of PSII, it remains to be determined whether salt stress induces damage to PSII directly.

The separate effects of salt stress on damage and repair have been examined in *Synechocystis* [10,78,79]. Salt stress, due to 0.5 M NaCl, inhibited the repair of photodamaged PSII but did not accelerate damage to PSII. Thus, it appears that the enhanced photoinhibition that was observed in earlier studies might have been due to the synergistic effects of light and salt

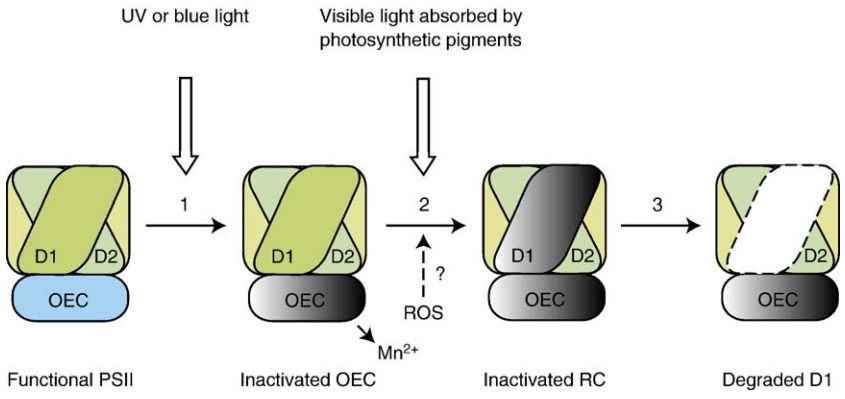

Fig. 3. A new model for the mechanism of photodamage to PSII. Photodamage to PSII occurs in two sequential steps. The oxygen-evolving complex (OEC) is inactivated primarily by light, in particular, light in the UV and blue regions of the spectrum, with accompanying release of manganese ions from the complex (arrow 1). Subsequently, the reaction center (RC) is inactivated by light absorbed by photosynthetic pigments (arrow 2). Impairment of the oxygen-evolving complex might allow the free access of oxygen molecules to the reaction-center chlorophyll P680 and the subsequent production of $^1O_2$. Thus, the possible involvement of ROS in damage to the reaction center remains to be examined (interrupted arrow). The damaged D1 protein undergoes proteolytic degradation (arrow 3).

stress, namely, damage by light and inhibition of repair by salt stress.

The labeling of proteins in *Synechocystis* in vivo revealed that the synthesis of the D1 protein de novo was markedly suppressed by salt stress due to 0.5 M NaCl [10,79]. Moreover, salt stress suppressed not only the synthesis of the D1 protein but also the synthesis of almost all other proteins [10,79]. Thus, it is likely that inhibition by salt stress of the repair of PSII is attributable to suppression of the synthesis of proteins de novo. Northern and immunoblotting analyses revealed that salt stress inhibits the synthesis of the D1 protein at both the transcriptional and the translational level [79]. Since the translational step was more severely inhibited than the transcriptional step under salt stress, it appears that a major target for inhibition by salt stress might be translation [79].

Salt stress limits the photosynthetic fixation of CO_2 by reducing the availability of CO_2 [80] or by inactivating the enzymes involved in the Calvin cycle, such as Rubisco [81]. When such conditions are combined with strong light, it is expected that the generation of ROS in the photosynthetic machinery is accelerated. As we have seen, the interruption of the Calvin cycle in light results in the inhibition of the synthesis of proteins de novo [70,71]. Thus, it appears that the apparent inhibition of translation by salt stress might be due to the action of ROS.

8. Reevaluation of the dissipation of thermal energy in relation to photoinhibition

Light energy absorbed by PSII is effectively eliminated by a combination of photochemical and non-photochemical quenching processes, which can be monitored in terms of chlorophyll fluorescence. The non-photochemical quenching (NPQ) process serves as a "safety valve" by dissipating excess energy as heat and preventing oxidative damage [82,83]. The dissipation of thermal energy requires PsbS, a chlorophyll-binding protein of PSII, and zeaxanthin, a carotenoid involved in the xanthophyll cycle [84,85]. In mutants of *Arabidopsis* defective in NPQ, PSII exhibited enhanced susceptibility to photoinhibition, while in transformants of *Arabidopsis* with overexpressed PsbS, PSII was more tolerant to photoinhibition [86]. These observations imply that NPQ might protect PSII from photoinhibition. However, studies of the effects of NPQ on photodamage to PSII using mutants of *Arabidopsis* defective in NPQ or artificial quenchers of chlorophyll fluorescence revealed that NPQ protects PSII from photodamage to lesser extents than it would be expected from the previously proposed mechanisms of photodamage, such as the "acceptor-side" mechanism [61,64,87,88]. These observations are consistent with the newly proposed mechanism of photodamage [33,34] because photodamage to PSII is not initiated by the transfer of excess excited energy from triplet-state chlorophyll to oxygen molecules but by the light-dependent impairment of the oxygen-evolving complex. Thus, NPQ appears to play a role in preventing the generation of ROS by reducing PSII-mediated electron transport rather than in protecting PSII from damage under strong light. The apparent protection by NPQ of PSII from photoinhibition might be due to prevention of the oxidative stress that inhibits the repair of PSII.

9. Conclusions and future perspectives

The separation of photodamage to PSII from the repair of photodamaged PSII has allowed us to clarify the nature of the photoinhibition of PSII. Photodamage to PSII is a purely light-dependent event that is caused primarily by the light-dependent impairment of the oxygen-evolving complex (Fig. 3). In this context, photodamage is an unavoidable and inherent feature of PSII and, thus, the rapid and efficient repair of PSII is necessary to compensate for the loss of function. The repair of PSII is very susceptible to inhibition by the ROS that are generated as a result of the photosynthetic transport of electrons and the transfer of energy. Thus, strong light has two distinct effects on PSII: it damages PSII directly and it inhibits the repair of PSII via production of ROS. The inhibition of repair is attributable to the inhibition by ROS of the synthesis de novo of proteins and, in particular, of the D1 protein, that are required for the repair of PSII. A primary target for inhibition by ROS is the elongation step of translation (Fig. 2).

We do not yet know the exact mechanisms whereby the oxygen-evolving complex and, most probably, the manganese cluster, is damaged by absorption of light in the UV and blue regions of the spectrum. We also do not know how the reaction center of PSII is damaged by light absorbed by chlorophylls and carotenoids after the impairment of the oxygen-evolving complex. The possible involvement of ROS in damage to the reaction center needs to be examined. The mechanisms responsible for the repair of the oxygen-evolving complex after light-induced impairment and, in particular, for the reassembly of the manganese cluster in vivo, remain to be clarified. The molecular mechanisms by which the translational machinery is inhibited by ROS are also far from being understood. The particular susceptibility of translational elongation to ROS may well provide a clue to the nature of the ROS-induced inhibition of translation. Moreover, attention should also be paid to the physiological importance of the sensitivity of the translational machinery to ROS since inhibition of the translational machinery influences not only photosynthesis but also other important physiological processes within the cell. Future studies should be directed towards a full characterization of the molecular mechanisms of photodamage to PSII and of the actions of ROS in the inhibition of translation.

Various types of environmental stress, including salt stress and low-temperature stress, enhance the photoinhibition of PSII. Recent studies have attributed such enhanced photoinhibition to the synergistic effects of the light-dependent damage and the inhibition of repair by salt stress or low-temperature stress, and they have also revealed that the synthesis of proteins de novo is markedly inhibited under such stress conditions [89]. It seems likely that salt stress or low-temperature stress suppresses the translational machinery indirectly via oxidative stress that is induced by these types of environmental stress through, for example, suppression of the Calvin cycle.

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