PCP
PLANT & CELL PHYSIOLOGY

# Overexpressed Superoxide Dismutase and Catalase Act Synergistically to Protect the Repair of PSII during Photoinhibition in *Synechococcus elongatus* PCC 7942

Penporn Sae-Tang[1], Yukako Hihara[1,2], Isao Yumoto[3], Yoshitake Orikasa[4], Hidetoshi Okuyama[5] and Yoshitaka Nishiyama[1,2,*]

[1]Department of Biochemistry and Molecular Biology, Graduate School of Science and Engineering, Saitama University, 255 Shimo-Okubo, Sakura-ku, Saitama, 338-8570 Japan
[2]Institute for Environmental Science and Technology, Saitama University, 255 Shimo-Okubo, Sakura-ku, Saitama, 338-8570 Japan
[3]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo, 062-8517 Japan
[4]Department of Food Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080-8555 Japan
[5]Laboratory of Environmental Molecular Biology, Faculty of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo, 060-0810 Japan
*Corresponding author: E-mail, nishiyama@molbiol.saitama-u.ac.jp; Fax, +81-48-858-3384.
(Received March 11, 2016; Accepted June 3, 2016)

**The repair of PSII under strong light is particularly sensitive to reactive oxygen species (ROS), such as the superoxide radical and hydrogen peroxide, and these ROS are efficiently scavenged by superoxide dismutase (SOD) and catalase. In the present study, we generated transformants of the cyanobacterium *Synechococcus elongatus* PCC 7942 that overexpressed an iron superoxide dismutase (Fe-SOD) from *Synechocystis* sp. PCC 6803; a highly active catalase (VktA) from *Vibrio rumoiensis*; and both enzymes together. Then we examined the sensitivity of PSII to photoinhibition in the three strains. In cells that overexpressed either Fe-SOD or VktA, PSII was more tolerant to strong light than it was in wild-type cells. Moreover, in cells that overexpressed both Fe-SOD and VktA, PSII was even more tolerant to strong light. However, the rate of photodamage to PSII, as monitored in the presence of chloramphenicol, was similar in all three transformant strains and in wild-type cells, suggesting that the overexpression of these ROS-scavenging enzymes might not protect PSII from photodamage but might protect the repair of PSII. Under strong light, intracellular levels of ROS fell significantly, and the synthesis de novo of proteins that are required for the repair of PSII, such as the D1 protein, was enhanced. Our observations suggest that overexpressed Fe-SOD and VktA might act synergistically to alleviate the photoinhibition of PSII by reducing intracellular levels of ROS, with resultant protection of the repair of PSII from oxidative inhibition.**

**Keywords:** Catalase • Oxidative stress • Photoinhibition • Photosystem II • Protein synthesis • Superoxide dismutase.

**Abbreviations:** Carboxy-$H_2$DCFDA, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; CBB, Coomassie Brilliant Blue; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase.

## Introduction

Photosynthesis is sensitive to various types of environmental stress, and strong light is one of the most significant environmental stressors. Exposure of photosynthetic organisms to strong light often results in the specific inactivation of PSII, and this phenomenon is referred to as photoinhibition of PSII (Powles 1984, Aro et al. 1993). In living cells, PSII is damaged by light and, almost simultaneously, it is repaired by a rapid repair system (Aro et al. 1993, Aro et al. 2005). Thus, photoinhibition results from the imbalance between the rate of the light-induced damage (photodamage) to PSII and the rate of repair of photodamaged PSII (Aro et al. 1993, Aro et al. 2005). Photoinhibition becomes apparent when the rate of photodamage exceeds the rate of repair. In order to understand the nature of photoinhibition, it is necessary to study the processes of photodamage and repair separately, and methods for the separate examination of these processes have been established in cyanobacteria and plants (Gombos et al. 1994, Wada et al. 1994, Moon et al. 1995).

Examination of photodamage and repair separately has revealed several new aspects of the mechanism of photoinhibition, in particular the role of reactive oxygen species (ROS) in the photoinhibition of PSII (Murata et al. 2012, Nishiyama and Murata 2014). When photosynthetic reactions are driven by light energy, ROS are produced as inevitable by-products by the photosynthetic machinery. The superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical (OH·) are produced as a result of the photosynthetic transport of electrons, while singlet oxygen ($^1O_2$) is produced as a result of the photosynthetic transfer of excitation energy via the triplet state of Chls (Asada 1999). The production of ROS is greatly stimulated under strong light (Asada 1999). Initially, these ROS, and in particular $^1O_2$, were considered to be the cause of

*Plant Cell Physiol.* 57(9): 1899–1907 (2016) doi:10.1093/pcp/pcw110, Advance Access publication on 21 June 2016, available online at www.pcp.oxfordjournals.org

photodamage to PSII (Vass et al. 1992, Hideg et al. 1994, Keren et al. 1997), but recent studies involving examinations of photoinhibition alone demonstrated that ROS act primarily by inhibiting the repair of PSII rather than by accelerating photodamage to PSII (Nishiyama et al. 2006, Nishiyama et al. 2011, Takahashi and Badger 2011, Murata et al. 2012). For example, in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*), photodamage to PSII was not accelerated but the repair of PSII was inhibited by increases in intracellular levels of electron transport-derived ROS, such as $O_2^-$ and $H_2O_2$. Increases in levels of ROS were achieved by addition of exogenous $H_2O_2$ and methyl viologen to cells and by suppression of the expression of genes for catalase and thioredoxin peroxidase (Nishiyama et al. 2001); by raising intracellular levels of $^1O_2$ via addition of exogenous photosensitizers, such as rose bengal and ethyl eosin, to cells (Nishiyama et al. 2004); and via suppression of expression of genes for enzymes involved in the synthesis of α-tocopherol (Inoue et al. 2011) and carotenoids, such as zeaxanthin and echinenone (Kusama et al. 2015). In contrast, the repair of PSII was protected by overexpression of the catalase VktA, derived from *Vibrio rumoiensis*, in the cyanobacterium *Synechococcus elongatus* PCC 7942 (hereafter referred to as *Synechococcus*) (Jimbo et al. 2013), and also by overexpression of catalase KatE, derived from *Escherichia coli*, in tobacco plants (Al-Taweel et al. 2007). However, the extent of the protection of the repair of PSII by overexpression of catalases in the above-cited studies was limited, and the ROS whose effects must be nullified to protect the repair of PSII remain to be identified.

The photosynthetic transport of electrons generates $O_2^-$ on the acceptor side of PSI, and this $O_2^-$ is rapidly converted to $H_2O_2$ by superoxide dismutase (SOD). $H_2O_2$ is then converted to $H_2O$ by a catalase or peroxidase (Fujii et al. 1990, Asada 1999). $H_2O_2$ can also be converted to OH·, the most toxic ROS, via the Fenton reaction in the presence of reduced metal ions, such as $Fe^{2+}$, which is generated via the reduction of $Fe^{3+}$ by $O_2^-$ (Halliwell and Gutteridge 1986). To prevent the generation and accumulation of these harmful ROS, ROS-scavenging enzymes play vital roles. SODs can be divided into four groups according to their metal cofactors, namely iron- (Fe-), manganese- (Mn-), copper/zinc- (Cu/Zn-) and nickel- (Ni-)SODs. The main SODs in cyanobacteria are Mn-SOD and Fe-SOD, which are encoded by the *sodA* and the *sodB* genes, respectively. The genomes of *Synechocystis* and *Synechococcus* encode only Fe-SOD (Priya et al. 2007), while many other cyanobacterial species have multiple SODs. Thus, it seems plausible that Fe-SOD might be particularly important among the four metal-SODs. Overexpression of *sodB* from *Synechocystis* in *E. coli* resulted in increased SOD activity and protection of cells from $O_2^-$-mediated oxidative stress (Bhattacharya et al. 2004). However, overexpression of Mn-SOD alone did not diminish the total intracellular level of ROS because enhancement of the ability to detoxify $O_2^-$ necessarily raises levels of $H_2O_2$ (Payton et al. 1997). Overexpression of both SOD and catalase/peroxidase should potentially resolve this problem. Indeed, overexpression of Mn-SOD and catalase in *Bifidobacterium longum* allowed this anaerobic bacterium to tolerate oxygenic growth as a result of an enhanced capacity to decompose $O_2^-$ and $H_2O_2$ (Zuo et al. 2014). In addition, overexpression of both Cu/Zn-SOD and ascorbate peroxidase in potato and tall fescue plants increased the tolerance of both plants to $H_2O_2$ and methyl viologen, as well as to high temperature (Tang et al. 2006, Lee et al. 2007).

In the present study, we examined the effects of overexpression of SOD and catalase, separately and together, on the photoinhibition of PSII in *Synechococcus*. We chose Fe-SOD from *Synechocystis* to avoid co-suppression of the expression of the *sodB* gene in the same organism and VktA as the catalase since its catalytic activity is 4.3- and 19-fold higher than that of bovine catalase and KatE of *E. coli*, respectively (Ichise et al. 2000, Yumoto et al. 2000). Under strong light, overexpression of both Fe-SOD and VktA protected PSII from photoinhibition by enhancing the repair of PSII, with decreases in levels of ROS and acceleration of the synthesis of the D1 protein, a core protein in the PSII reaction center.

## Results

### Overexpression of Fe-SOD and VktA

The *sodB* gene from *Synechocystis* and the *vktA* gene from *Vibrio rumoiensis*, together with the strong constitutive promoter conII, were inserted into a neutral site in the genome of *Synechococcus* by homologous recombination (**Fig. 1A**). The resultant transformants were designated *sodB* and *vktA*, respectively. The combination of *sodB* and *vktA* genes together, with individual conII promoters, was also inserted into the genome for overexpression of both Fe-SOD and VktA together (**Fig. 1A**). The resultant transformant was designated *sodBvktA*. Complete segregation was confirmed by PCR with forward and reverse primers (**Supplementary Table S1**) that flanked the inserted sequences and genomic DNA as template (**Fig. 1B**).

When proteins in extracts from *sodB* and *sodBvktA* cells were separated by SDS–PAGE and stained by Coomassie Brilliant Blue (CBB), an intense band was detected at 23 kDa, which corresponds to the molecular mass (22.5 kDa) of Fe-SOD (**Fig. 2A**). In the case of *vktA* and *sodBvktA* cells, an intense band was detected at 60 kDa, which corresponds to the molecular mass (57.3 kDa) of VktA (**Fig. 2A**). Overexpression of Fe-SOD was confirmed by an in-gel assay of SOD, which showed that the activity of SOD had approximately doubled (**Fig. 2B**). Overexpression of VktA was confirmed immunologically with VktA-specific antibodies (**Fig. 2C**).

### Overexpression of Fe-SOD and VktA alleviates the photoinhibition of PSII

We examined the effects of overexpression of Fe-SOD and VktA on photoinhibition of PSII. When wild-type cells were exposed to strong light at 1,500 μmol photons $m^{-2}$ $s^{-1}$ at 32°C, the activity of PSII declined, as is typical during the photoinhibition of PSII (**Fig. 3A**). However, the sensitivity of PSII to strong light differed among wild-type and transformed cells. PSII in *vktA* cells was more resistant to strong light than that in wild-type cells; PSII in *sodB* cells was more resistant than that in *vktA* cells;

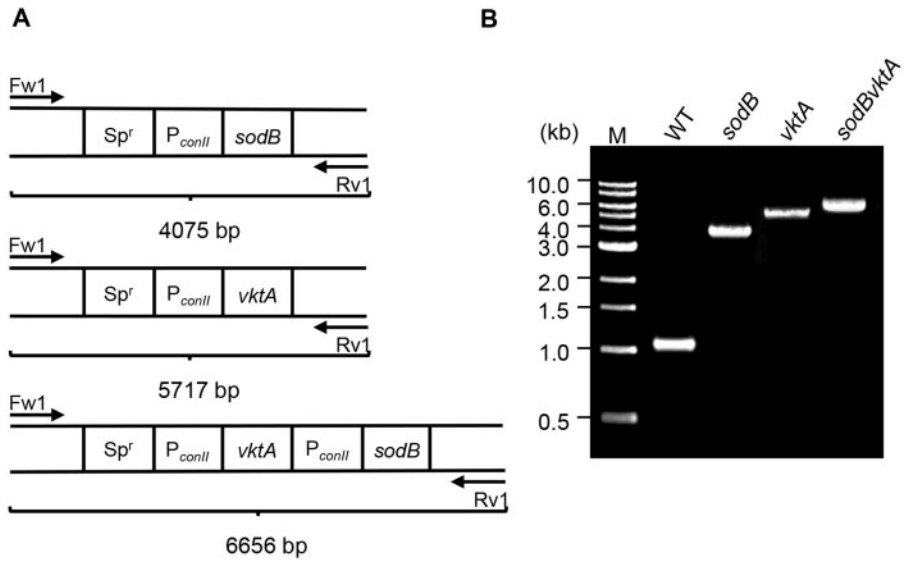


**Fig. 1** Generation of transformants of *Synechococcus* and confirmation of the segregation of genomes. (A) Schematic illustration of the constructs for overexpression of *sodB* and *vktA*, separately and together, in *Synechococcus*. Each gene with the strong constitutive promoter conII ($P_{conII}$) and a spectinomycin resistance gene cassette ($Sp^r$) was incorporated into a neutral site of the genome. (B) Analysis by PCR to confirm the complete replacement of the wild-type gene by the appropriate insert. DNA fragments were amplified with specific primers and genomic DNA from wild-type (WT) and transformed cells. Arrows in (A) indicate the positions and directions of forward (Fw1) and reverse (Rv1) primers for PCR. M, molecular size markers.

and PSII in *sodBvktA* cells was more resistant than that in *sodB* cells (Fig. 3A). Thus, it appeared that the overexpression of either Fe-SOD or VktA protected PSII from photoinhibition of PSII, while the overexpression of both Fe-SOD and VktA together protected PSII from photoinhibition to an even greater extent. In contrast, when cells were exposed to strong light at the same intensity in the presence of chloramphenicol, which blocks the repair of PSII, the activity of PSII declined at the same rate in the wild type and all strains of transformed cells (Fig. 3B). These observations suggested that the overexpression of Fe-SOD and VktA did not protect PSII from photodamage but did protect the repair of PSII, with resultant protection of PSII against photoinhibition.

## Overexpression of Fe-SOD and VktA protects the repair of PSII in the presence of methyl viologen

Methyl viologen facilitates the transfer of electrons from the acceptor side of PSI to molecular oxygen, with resultant production of excess $O_2^-$ within cells. When cells were exposed to strong light at 1,500 μmol photons $m^{-2}$ $s^{-1}$ at 32°C in the presence of 2 μM methyl viologen, the activity of PSII declined more rapidly than the decline in its absence in all strains of cells. However, the protective effects of overexpression of Fe-SOD and VktA were still apparent (Fig. 4A). PSII in *vktA* cells was as resistant to strong light as the PSII in *sodB* cells, and PSII in *sodBvktA* cells was more resistant than the PSII in *sodB* or *vktA* cells (Fig. 4A). In contrast, the rate of photodamage to PSII, as measured in the presence of chloramphenicol, did not differ among wild-type and singly or doubly transformed cells (Fig. 4B), suggesting that the overexpression of Fe-SOD and VktA protected the repair of PSII in the presence of methyl viologen. Furthermore, rates of photodamage to PSII in the presence of methyl viologen were the same as those in its absence (Fig. 3B), suggesting that ROS might not accelerate photodamage to PSII but might inhibit the repair of PSII, as shown previously (Nishiyama et al. 2001).

## Overexpression of Fe-SOD and VktA depresses intracellular levels of $H_2O_2$ and related ROS

To examine the effects of overexpression of Fe-SOD and VktA on levels of ROS under strong light, we monitored levels of ROS using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-$H_2$DCFDA). Intracellular esterases convert this compound to 2′,7′-dichlorodihydrofluorescein, which reacts with ROS that include $H_2O_2$, hydroxyl and peroxyl radicals, and peroxynitrite, but not with $O_2^-$, with resultant emission of fluorescence (Hakkila et al. 2014). Since this indicator is sensitive to strong light at 1,500 μmol photons $m^{-2}$ $s^{-1}$, we exposed cells to light at 500 μmol photons $m^{-2}$ $s^{-1}$ at 32°C. In wild-type cells, levels of the above-mentioned ROS rose as illumination was prolonged (Fig. 5A). In *sodB* cells, levels of ROS rose at a higher rate than those in wild-type cells (Fig. 5A). This phenomenon might have been due to the accumulation of $H_2O_2$ via the accelerated decomposition of $O_2^-$. In contrast, the production of ROS was depressed in *vktA* cells and, to a greater extent, in *sodBvktA* cells (Fig. 5A). We also observed that the depressed production of ROS in *sodBvktA* cells was even under illumination from a standard growth light at 70 μmol photons $m^{-2}$ $s^{-1}$ for 30 min (Fig. 5A). Illumination in the presence of methyl viologen enhanced this trend, with higher production of ROS in wild-type and *sodB* cells and lower production of ROS in *vktA* and *sodBvktA* cells (Fig. 5B). Thus, overexpression of Fe-SOD alone

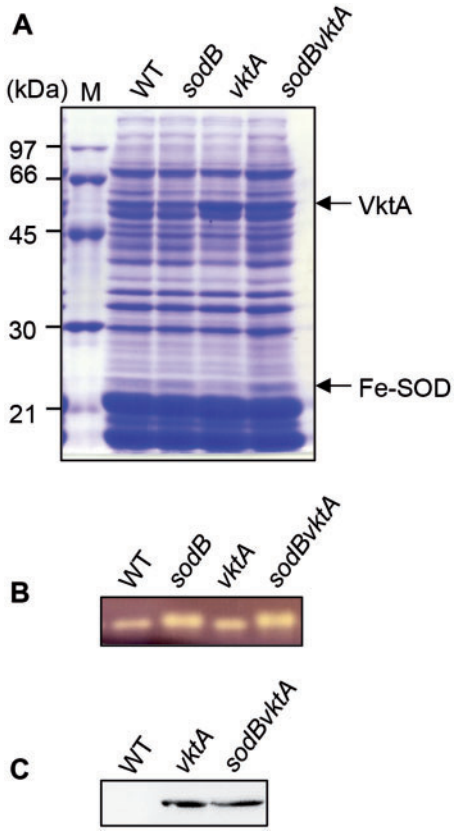


**Fig. 2** Detection of the products of *sodB* and *vktA* in *Synechococcus* cells. (A) Soluble proteins in wild-type and transformed cells. Soluble fractions were isolated from cells, and the proteins in these fractions were analyzed. Proteins (20 μg per lane) were separated by SDS–PAGE on a 12% polyacrylamide gel and stained with CBB. (B) In-gel assay of the activity of SOD. Proteins (20 μg per lane) were separated on a 10% non-denaturing gel and the activity of SOD was detected as described in the Materials and Methods. (C) Detection of VktA. Proteins (20 μg per lane) were separated by SDS–PAGE on a 12% polyacrylamide gel and VktA was detected immunologically. M, molecular size markers.

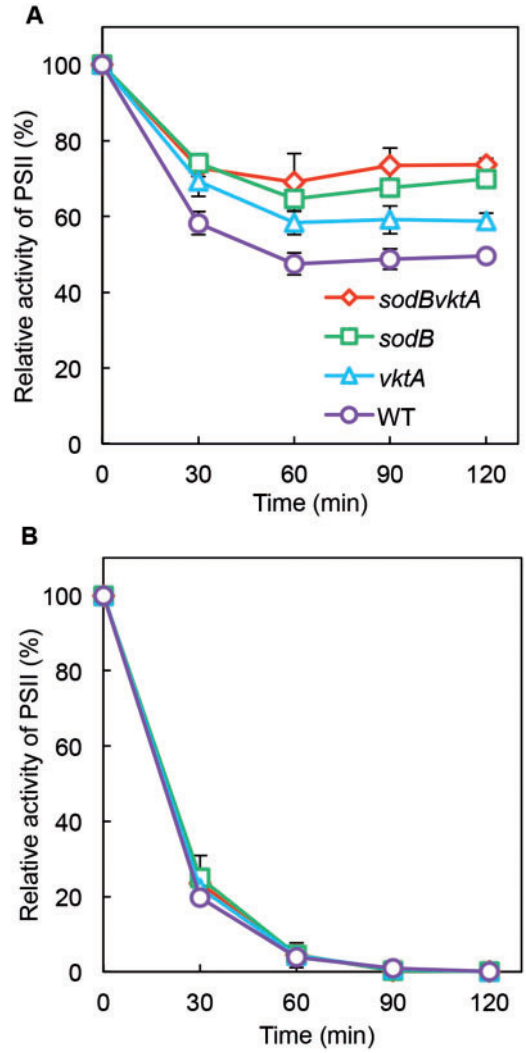


**Fig. 3** Effects of the overexpression of Fe-SOD, VktA and Fe-SOD plus VktA on the photoinhibition of PSII in *Synechococcus*. Cells were incubated at 32°C under strong light at 1,500 μmol photons $m^{-2}$ $s^{-1}$ with standard aeration in the absence of chloramphenicol (A) and in its presence (B). The activity of PSII was monitored in terms of the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone as the electron acceptor. The activities taken as 100% for wild-type, *sodB*, *vktA* and *sodBvktA* cells were 1,600 ± 120, 1,690 ± 80, 1,560 ± 80 and 1,670 ± 150 mmol $O_2$ $mg^{-1}$ Chl $h^{-1}$, respectively. Values are means ± SD (bars) of results from at least three independent experiments. In this and other figures, the absence of a bar indicates that the SD falls within the symbol.

did not depress levels of $H_2O_2$ and related ROS under strong light, whereas overexpression of both Fe-SOD and VktA effectively depressed levels of these ROS. We attempted to monitor changes in intracellular levels of $O_2^-$ using nitroblue tetrazolium (NBT), a specific indicator of $O_2^-$, under strong light, but we failed to detect $O_2^-$ because of the high background due to phycobilisomes around the absorption peak of NBT whose profile changes upon the reaction with $O_2^-$.

## Overexpression of Fe-SOD and VktA enhances the synthesis of the D1 protein under strong light

The repair of PSII requires the synthesis de novo of the D1 protein, a core protein that constitutes the reaction center of PSII. To examine the effects of overexpression of Fe-SOD and VktA on the synthesis of the D1 protein de novo under strong light, we exposed cells to light at 1,500 μmol photons $m^{-2}$ $s^{-1}$ at 25°C in the presence of $^{35}S$-labeled methionine and cysteine and monitored radiolabeled proteins in thylakoid membranes on a urea-containing polyacrylamide gel. Proteins on the gel were also stained with CBB as loading controls (**Supplementary Fig. S1**). The overexpression of Fe-SOD and VktA affected the rate of synthesis of the D1 protein under strong light. Compared with the rate in wild-type cells, the rate of synthesis was slightly higher in *vktA* cells; about 1.4-fold higher in *sodB* cells; and about twice as high in *sodBvktA* cells (**Fig. 6A**). Moreover, not only the synthesis of the D1 protein but also the synthesis of many other proteins in thylakoid membranes was enhanced in all three transformed cells (**Fig. 6A**). We quantified the entire complement of labeled proteins in thylakoid membranes by liquid scintillation counting. Levels of proteins that were newly synthesized over the course of 30 min under strong light in *sodB*, *vktA* and *sodBvktA* cells were higher than those in wild-type cells by factors

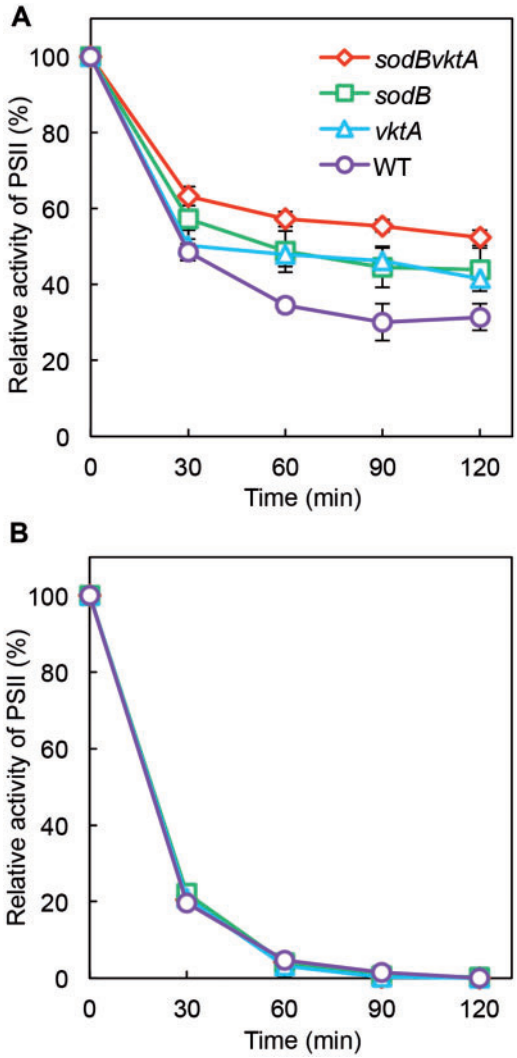


**Fig. 4** Effects of the overexpression of Fe-SOD, VktA and Fe-SOD plus VktA on the photoinhibition of PSII in the presence of 2 μM methyl viologen in *Synechococcus*. Cells were incubated at 32°C under strong light at 1,500 μmol photons $m^{-2}$ $s^{-1}$ with standard aeration in the absence of chloramphenicol (A) and in its presence (B). Values are means ± SD (bars) of results from at least three independent experiments.

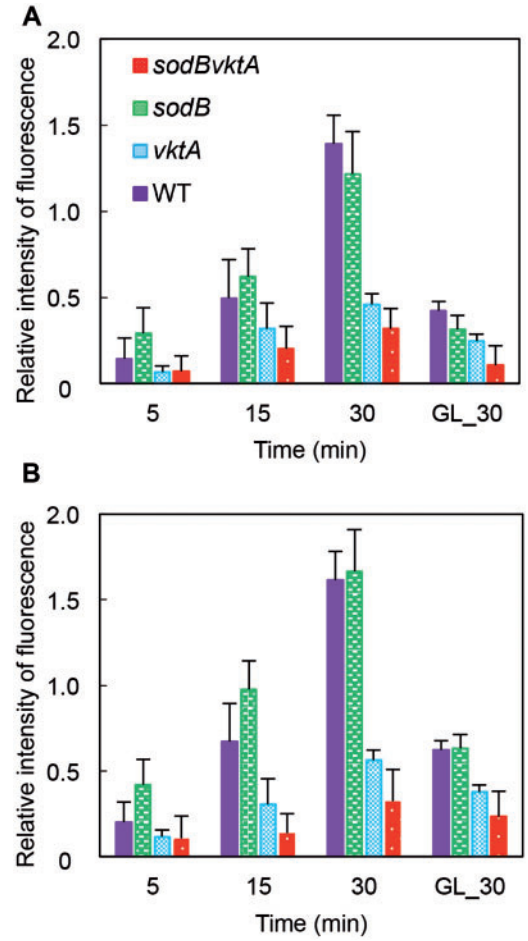


**Fig. 5** Production of $H_2O_2$ and related ROS during illumination of cells. (A) Cells were illuminated under strong light at 500 μmol photons $m^{-2}$ $s^{-1}$ for the indicated times or under the standard growth light at 70 μmol photons $m^{-2}$ $s^{-1}$ for 30 min (GL_30) in the presence of the fluorescence indicator carboxy-$H_2$DCFDA. (B) Levels of $H_2O_2$ and related ROS were monitored under the same conditions as in (A) but in the presence of 2 μM methyl viologen. Values are means ± SD (bars) of results from three independent experiments.

of approximately 1.4, 1.1 and 2, respectively (**Fig. 6B**), showing the same trend as that observed in the synthesis de novo of the D1 protein. Thus, under strong light, overexpression of both Fe-SOD and VktA accelerated not only the synthesis of the D1 protein but also the synthesis of the other proteins in thylakoid membranes.

## Discussion

### Roles of SOD and catalase in the protection of PSII from photoinhibition

The present study showed that overexpression of Fe-SOD and VktA in *Synechococcus* alleviated the photoinhibition of PSII, indicating that SOD and catalase protect PSII from photoinhibition. Overexpression of VktA alone alleviated the photoinhibition of PSII, but the protective effect was limited to approximately 10% (**Fig. 3**), as observed previously by Jimbo et al. (2013). The minimal effect of VktA on photoinhibition might have been due to the accumulation of $O_2^-$ in VktA-overexpressing cells under strong light, even though intracellular levels of $H_2O_2$ fell significantly (**Fig. 5**). Overexpression of Fe-SOD alone alleviated the photoinhibition of PSII to a greater extent than overexpression of VktA alone (**Fig. 3**). The more pronounced effect might be explained by the effective decomposition of $O_2^-$ in Fe-SOD-overexpressing cells, even though intracellular levels of harmful $H_2O_2$ did increase (**Fig. 5**). Overexpression of both Fe-SOD and VktA together alleviated the photoinhibition of PSII much more effectively (**Fig. 3**), indicating that decreases in levels of both $O_2^-$ and $H_2O_2$ efficiently protected PSII from photoinhibition. Although we were unable to compare the individual effects of $O_2^-$ and $H_2O_2$ quantitatively, it is likely that both ROS are able to increase the extent of photoinhibition of PSII. In addition, $H_2O_2$ can be converted to OH·, the most toxic ROS, via the Fenton reaction in the

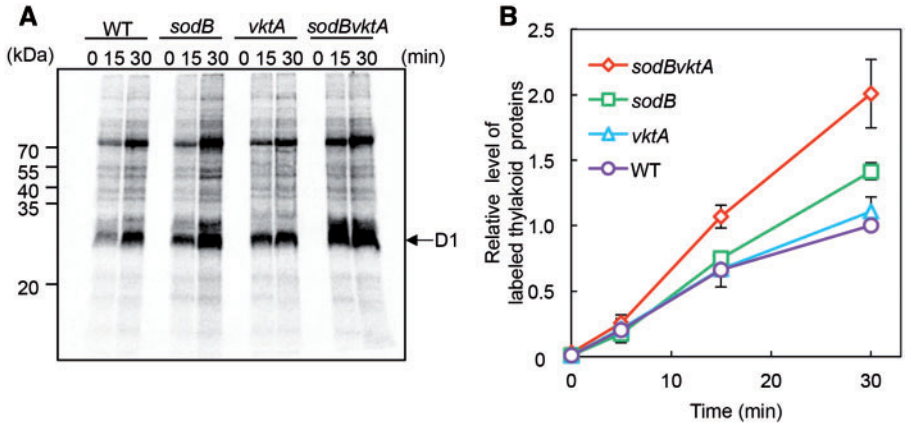


**Fig. 6** Effects of the expression of Fe-SOD, VktA and Fe-SOD plus VktA on the synthesis of the D1 protein de novo in *Synechococcus*. Proteins in wild-type (WT) and transformed cells were pulse-labeled with $^{35}$S-labeled methionine plus cysteine during incubation of cells at 25°C, for the indicated times, under strong light at 1,500 μmol photons $m^{-2}$ $s^{-1}$. (A) Radiogram of proteins from thylakoid membranes. (B) Levels of unfractionated labeled proteins in thylakoid membranes were determined by liquid scintillation counting. Values are means ± SD (bars) of results from at least three independent experiments.

presence of reduced metal ions, such as $Fe^{2+}$, which is generated by the reduction of $Fe^{3+}$ by $O_2^-$ (Keyer and Imlay 1996, McCormick et al. 1998). Thus, suppression of the production of both $O_2^-$ and $H_2O_2$ should also mitigate oxidative stress by depressing the generation of OH·.

The addition of methyl viologen to cells stimulated the photoinhibition of PSII in all four strains examined (**Fig. 4**). In the presence of this compound, the protective effect of overexpression of VktA was almost equivalent to that of the overexpression of Fe-SOD. It seems likely that enhanced production of $O_2^-$ in the presence of methyl viologen might have increased the accumulation of $H_2O_2$ and that the capacity to decompose $H_2O_2$ might, in turn, have become a rate-limiting step. Therefore, the effect of overexpression of VktA might have been enhanced, as compared with that of the overexpression of Fe-SOD that resulted in more pronounced increases in levels of $H_2O_2$ (**Fig. 5**). In addition, the strong protective effect of overexpression of both Fe-SOD and VktA together was probably due to synergistic abilities of these enzymes to scavenge both $O_2^-$ and $H_2O_2$.

## Actions of ROS in the photoinhibition of PSII

The overexpression of Fe-SOD and VktA did not affect photodamage to PSII but enhanced the repair of PSII, indicating that SOD and catalase might not protect PSII from photodamage but might, rather, protect the repair of PSII (**Fig. 3**). Moreover, the addition of methyl viologen to cells did not accelerate the rate of photodamage to PSII, as observed previously (Nishiyama et al. 2001). These observations support our hypothesis that ROS act primarily by inhibiting the repair of PSII and not by damaging PSII directly (Nishiyama et al. 2001, Nishiyama et al. 2004, Nishiyama et al. 2011). They are also consistent with the 'two-step mechanism' of photodamage to PSII. In the two-step mechanism, photodamage to PSII occurs in two steps: primary damage occurs at the oxygen-evolving complex, most probably at the manganese cluster, via absorption of UV and blue light; and secondary damage occurs at the reaction center via absorption of visible light that has been absorbed by Chls (Hakala et al. 2005, Ohnishi et al. 2005). According to this mechanism, ROS are not the primary cause of photodamage to PSII or, at least, they are not responsible for primary damage to PSII (Nishiyama et al. 2006).

## Role of SOD and catalase in the protection of protein synthesis from ROS

Under strong light, overexpression of either Fe-SOD or VktA enhanced the synthesis de novo of the D1 protein, which is required for the repair of PSII, while the overexpression of both Fe-SOD and VktA together enhanced the synthesis of D1 to a much greater extent (**Fig. 6**). The synergistic effects of the two enzymes might again be explained by decreased levels of both $O_2^-$ and $H_2O_2$, as well as by the resultant decrease in levels of OH·. Furthermore, we observed that not only was the synthesis of the D1 protein enhanced but the synthesis of almost all the proteins in thylakoid membranes was also enhanced. This observation suggests that the protein synthetic machinery might itself have been protected from damage by ROS.

The particular sensitivity of the protein synthetic machinery to ROS was demonstrated in studies of the effects of elevated levels of ROS on protein synthesis in *Synechocystis* (Nishiyama et al. 2001, Nishiyama et al. 2004). Analysis of polysomes in *Synechocystis* revealed that ROS inhibit the synthesis of the D1 protein de novo at the elongation step of translation (Nishiyama et al. 2001, Nishiyama et al. 2004). Moreover, within the translational machinery, elongation factors EF-G and EF-Tu were identified as the targets of ROS in *Synechocystis*. In *Synechocystis*, EF-G is inactivated via the oxidation, by ROS, of two specific cysteine residues, with subsequent formation of an intramolecular disulfide bond (Kojima et al. 2007, Kojima et al. 2009), while EF-Tu is inactivated via oxidation of the single cysteine residue (Cys82) with subsequent formation of an intermolecular disulfide bond and sulfenic acid (Yutthanasirikul et al. 2016). The particular sensitivity of EF-G to ROS has also been demonstrated in *E. coli* (Nagano et al. 2012, Nagano et al. 2015). It remains to be determined whether overexpression of Fe-SOD and VktA can suppress the oxidation of

these elongation factors under strong light, thereby protecting protein synthesis from ROS-induced inhibition.

## Conclusions and perspectives

Overexpression of both Fe-SOD and VktA mitigates the photoinhibition of PSII by protecting the repair of PSII rather than by protecting PSII from photodamage. Such protection might be achieved by the synergistic effects of efficient scavenging of both $O_2^-$ and $H_2O_2$ with the consequent protection, from oxidative damage, of the synthesis of the proteins that are required for the repair of PSII. Reinforcement of the capacity to scavenge $O_2^-$ and $H_2O_2$ with Fe-SOD and VktA might allow improvements in the tolerance of the photosynthetic machinery to strong light, as well as to other abiotic stressors that engender oxidative stress.

# Materials and Methods

## Cells and culture conditions

Wild-type cells and transformants of *Synechococcus elongatus* PCC 7942 that expressed *sodB* and *vktA* were grown photoautotrophically at 32°C in liquid BG11 medium under light at 70 μmol photons $m^{-2}$ $s^{-1}$, with aeration by sterile air that contained 1% (v/v) $CO_2$, as described previously (Kusama et al. 2015). Cells in cultures with an optical density at 730 nm of 1.0 ± 0.1 were used for assays, unless otherwise noted.

## Generation of transformants

A DNA fragment containing the *sodB* (*slr1516*) gene was amplified from the genomic DNA of *Synechocystis* sp. PCC 6803 with appropriated primers (**Supplementary Table S1**). A DNA fragment containing the *vktA* gene and its upstream region (Ichise et al. 2000) from *V. rumoiensis* S-1$^T$ was obtained as described previously (Jimbo et al. 2013). The ends of the DNA fragments were blunted and fragments were inserted into the *Sma*I site of the pAM1044 vector, which was designed to incorporate a gene of interest at a neutral site in the genome of *Synechococcus* and to express the gene under the strong constitutive promoter conII (originally provided by Professor Susan S. Golden, UC San Diego) (Deshnium et al. 1995). For overexpression of both *sodB* and *vktA* together, the plasmid was designed to include a conII promoter in the 5′ upstream regions of both *vktA* and *sodB* in tandem. The resultant plasmids were used to transform wild-type *Synechococcus* by homologous recombination.

## Expression of *sodB* and *vktA*

The successful incorporation of the *sodB*, *vktA* and *sodBvktA* genes into all the chromosomal copies of the genome was confirmed by PCR with appropriate primers (**Supplementary Table S1**). Cells were disrupted with glass beads, and proteins in the soluble fraction were fractionated by SDS–PAGE on a 12% polyacrylamide gel, with subsequent staining with CBB and immunological detection with VktA-specific antibodies, as described previously (Jimbo et al. 2013). To evaluate the activity of Fe-SOD, proteins were fractionated at 4°C by PAGE on a 10% non-denaturing gel. After electrophoresis, the gel was soaked for 20 min in a solution of 26 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and 25 μM riboflavin, and then stained by incubation with 2.5 mM NBT for a further 20 min. The gel was illuminated with white light at 30 μmol photons $m^{-2}$ $s^{-1}$ to initiate the chemical reaction, and illumination was continued until transparent bands appeared (Beauchamp and Fridovich 1971).

## Assay of the photoinhibition of PSII

Cells were exposed to light at 1,500 μmol photons $m^{-2}$ $s^{-1}$ at 32°C for the designated times to induce the photoinhibition of PSII, as described previously (Kusama et al. 2015). For assays of photodamage, chloramphenicol was added to the suspension of cells at a final concentration of 200 mg $ml^{-1}$ just before the onset of illumination. The activity of PSII was measured at 32°C in terms of the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone and 1 mM $K_3Fe(CN)_6$ with a Clark-type oxygen electrode (Hansatech Instruments).

## Determination of intracellular levels of ROS

Intracellular levels of ROS were measured with the fluorescence indicator carboxy-$H_2$DCFDA (Invitrogen), as described previously (Hakkila et al. 2014). Aliquots of 1 ml of cell suspension were incubated with 15 μM carboxy-$H_2$DCFDA at 32°C for 1 h in darkness. After cells had been washed and suspended in 0.5 ml of fresh BG11 medium, the suspension was exposed to strong light at 500 μmol photons $m^{-2}$ $s^{-1}$ or to the standard growth light at 70 μmol photons $m^{-2}$ $s^{-1}$ at 32°C for the designated times. The fluorescence emitted from the reaction product, 2′,7′-dichlorofluorescein, upon excitation at 485 nm was detected at 520 nm with a FLUOstar OPTIMA system (BMG Labtech). All data were normalized by reference to a peak of autofluorescence at 680 nm.

## Labeling of proteins in vivo

For pulse labeling of proteins, 30 ml of cell culture were incubated at 25°C in light at 1,500 μmol photons $m^{-2}$ $s^{-1}$ for 30 min in the presence of 240 kBq $ml^{-1}$ $^{35}$S-labeled methionine plus cysteine (EasyTagTM EXPRE35S $^{35}$S; PerkinElmer), as described previously (Nishiyama et al. 2004). Aliquots of 7 ml of each suspension were withdrawn at the designated times for analysis of proteins. Labeling was terminated by the addition of non-radioactive methionine and cysteine to a final concentration of 2 mM each with immediate cooling of samples on ice. Thylakoid membranes were isolated from cells as described previously (Nishiyama et al. 2004), and proteins from thylakoid membranes that corresponded to 10 μg of Chl were separated by SDS–PAGE on a 12.5% polyacrylamide gel that contained 6 M urea. Labeled proteins on the gel were visualized with an imaging analyzer (FLA-7000; Fujifilm Life Science). Levels of unfractionated labeled proteins in thylakoid membranes were also quantitated by liquid scintillation counting, as described previously (Kojima et al. 2007).

# Supplementary data

**Supplementary data** are available at PCP online.

# Funding

This work was supported by the Japan Society for the Promotion of Science [a Grant-in-Aid for Scientific Research (No. 15K07097 to Y.N.)].

# Acknowledgments

The authors thank Mr. Tomohisa Niimi (Saitama University) for his skilled technical assistance.

# Disclosures

The authors have no conflicts of interest to declare.

# References

Al-Taweel, K., Iwaki, T., Yabuta, Y., Shigeoka, S., Murata, N. and Wadano, A. (2007) A bacterial transgene for catalase protects translation of D1 protein during exposure of salt-stressed tobacco leaves to strong light. *Plant Physiol.* 145: 258–265.

Aro, E.M., Suorsa, M., Rokka, A., Allahverdiyeva, Y., Paakkarinen, V., Saleem, A., et al. (2005) Dynamics of photosystem II: a proteomic approach to thylakoid protein complexes. *J. Exp. Bot.* 56: 347–356.

Aro, E.M., Virgin, I. and Andersson, B. (1993) Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochim. Biophys. Acta* 1143: 113–134.

Asada, K. (1999) The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50: 601–639.

Beauchamp, C. and Fridovich, I. (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44: 276–287.

Bhattacharya, J., GhoshDastidar, K., Chatterjee, A., Majee, M. and Majumder, A.L. (2004) *Synechocystis* Fe superoxide dismutase gene confers oxidative stress tolerance to *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 316: 540–544.

Deshnium, P., Los, D., Hayashi, H., Mustardy, L. and Murata, N. (1995) Transformation of *Synechococcus* with a gene for choline oxidase enhances tolerance to salt stress. *Plant Mol. Biol.* 29: 897–907.

Fujii, T., Yokoyama, E., Inoue, K. and Sakurai, H. (1990) The sites of electron donation of photosystem I to methyl viologen. *Biochim. Biophys. Acta* 1015: 41–48.

Gombos, Z., Wada, H. and Murata, N. (1994) The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance. *Proc. Natl. Acad. Sci. USA* 91: 8787–8791.

Hakala, M., Tuominen, I., Keränen, M., Tyystjärvi, T. and Tyystjärvi, E. (2005) Evidence for the role of the oxygen-evolving manganese complex in photoinhibition of photosystem II. *Biochim. Biophys. Acta* 1706: 68–80.

Hakkila, K., Antal, T., Rehman, A.U., Kurkela, J., Wada, H., Vass, I., et al. (2014) Oxidative stress and photoinhibition can be separated in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochim. Biophys. Acta* 1837: 217–225.

Halliwell, B. and Gutteridge, J.M.C. (1986) Iron and free radical reactions: two aspects of antioxidant protection. *Trends Biochem. Sci.* 11: 372–375.

Hideg, É., Spetea, C. and Vass, I. (1994) Singlet oxygen and free radical production during acceptor- and donor-side-induced photoinhibition: studies with spin trapping EPR spectroscopy. *Biochim. Biophys. Acta* 1186: 143–152.

Ichise, N., Morita, N., Kawasaki, K., Yumoto, I. and Okuyama, H. (2000) Gene cloning and expression of the catalase from the hydrogen peroxide-resistant bacterium *Vibrio rumoiensis* S-1 and its subcellular localization. *J. Biosci. Bioeng.* 90: 530–534.

Inoue, S., Ejima, K., Iwai, E., Hayashi, H., Appel, J., Tyystjarvi, E., et al. (2011) Protection by alpha-tocopherol of the repair of photosystem II during photoinhibition in *Synechocystis* sp. PCC 6803. *Biochim. Biophys. Acta* 1807: 236–241.

Jimbo, H., Noda, A., Hayashi, H., Nagano, T., Yumoto, I., Orikasa, Y., et al. (2013) Expression of a highly active catalase VktA in the cyanobacterium *Synechococcus elongatus* PCC 7942 alleviates the photoinhibition of photosystem II. *Photosynth. Res.* 117: 509–515.

Keren, N., Berg, A., van Kan, P.J.M., Levanon, H. and Ohad, I. (1997) Mechanism of photosystem II photoinactivation and D1 protein degradation at low light: the role of back electron flow. *Proc. Natl. Acad. Sci. USA* 94: 1579–1584.

Keyer, K. and Imlay, J.A. (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proc. Natl. Acad. Sci. USA* 93: 13635–13640.

Kojima, K., Motohashi, K., Morota, T., Oshita, M., Hisabori, T., Hayashi, H., et al. (2009) Regulation of translation by the redox state of elongation factor G in the cyanobacterium *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* 284: 18685–18691.

Kojima, K., Oshita, M., Nanjo, Y., Kasai, K., Tozawa, Y., Hayashi, H., et al. (2007) Oxidation of elongation factor G inhibits the synthesis of the D1 protein of photosystem II. *Mol. Microbiol.* 65: 936–947.

Kusama, Y., Inoue, S., Jimbo, H., Takaichi, S., Sonoike, K., Hihara, Y., et al. (2015) Zeaxanthin and echinenone protect the repair of photosystem II from inhibition by singlet oxygen in *Synechocystis* sp. PCC 6803. *Plant Cell Physiol.* 56: 906–916.

Lee, S.H., Ahsan, N., Lee, K.W., Kim, D.H., Lee, D.G., Kwak, S.S., et al. (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. *J. Plant Physiol.* 164: 1626–1638.

McCormick, M.L., Buettner, G.R. and Britigan, B.E. (1998) Endogenous superoxide dismutase levels regulate iron-dependent hydroxyl radical formation in *Escherichia coli* exposed to hydrogen peroxide. *J. Bacteriol.* 180: 622–625.

Moon, B.Y., Higashi, S., Gombos, Z. and Murata, N. (1995) Unsaturation of the membrane lipids of chloroplasts stabilizes the photosynthetic machinery against low-temperature photoinhibition in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA* 92: 6219–6223.

Murata, N., Allakhverdiev, S.I. and Nishiyama, Y. (2012) The mechanism of photoinhibition in vivo: re-evaluation of the roles of catalase, alpha-tocopherol, non-photochemical quenching, and electron transport. *Biochim. Biophys. Acta* 1817: 1127–1133.

Nagano, T., Kojima, K., Hisabori, T., Hayashi, H., Morita, E.H., Kanamori, T., et al. (2012) Elongation factor G is a critical target during oxidative damage to the translation system of *Escherichia coli*. *J. Biol. Chem.* 287: 28697–28704.

Nagano, T., Yutthanasirikul, R., Hihara, Y., Hisabori, T., Kanamori, T., Takeuchi, N., et al. (2015) Oxidation of translation factor EF-G transiently retards the translational elongation cycle in *Escherichia coli*. *J. Biochem.* 158: 165–172.

Nishiyama, Y., Allakhverdiev, S.I. and Murata, N. (2006) A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II. *Biochim. Biophys. Acta* 1757: 742–749.

Nishiyama, Y., Allakhverdiev, S.I. and Murata, N. (2011) Protein synthesis is the primary target of reactive oxygen species in the photoinhibition of photosystem II. *Physiol. Plant.* 142: 35–46.

Nishiyama, Y., Allakhverdiev, S.I., Yamamoto, H., Hayashi, H. and Murata, N. (2004) Singlet oxygen inhibits the repair of photosystem II by suppressing the translation elongation of the D1 protein in *Synechocystis* sp. PCC 6803. *Biochemistry* 43: 11321–11330.

Nishiyama, Y. and Murata, N. (2014) Revised scheme for the mechanism of photoinhibition and its application to enhance the abiotic stress tolerance of the photosynthetic machinery. *Appl. Microbiol. Biotechnol.* 98: 8777–8796.

Nishiyama, Y., Yamamoto, H., Allakhverdiev, S.I., Inaba, M., Yokota, A. and Murata, N. (2001) Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. *EMBO J.* 20: 5587–5594.

Ohnishi, N., Allakhverdiev, S.I., Takahashi, S., Higashi, S., Watanabe, M., Nishiyama, Y., et al. (2005) Two-step mechanism of photodamage to photosystem II: step 1 occurs at the oxygen-evolving complex and step 2 occurs at the photochemical reaction center. *Biochemistry* 44: 8494–8499.

Payton, P., Allen, R., Trolinder, N. and Scott Holaday, A. (1997) Over-expression of chloroplast-targeted Mn superoxide dismutase in cotton (*Gossypium hirsutum* L., cv. Coker 312) does not alter the reduction of photosynthesis after short exposures to low temperature and high light intensity. *Photosynth. Res.* 52: 233–244.

Powles, S.B. (1984) Photoinhibition of photosynthesis induced by visible light. *Annu. Rev. Plant Physiol.* 35: 15–44.

Priya, B., Premanandh, J., Dhanalakshmi, R.T., Seethalakshmi, T., Uma, L., Prabaharan, D., et al. (2007) Comparative analysis of cyanobacterial superoxide dismutases to discriminate canonical forms. *BMC Genomics* 8: 435.

Takahashi, S. and Badger, M.R. (2011) Photoprotection in plants: a new light on photosystem II damage. *Trends Plant Sci.* 16: 53–60.

Tang, L., Kwon, S.Y., Kim, S.H., Kim, J.S., Choi, J.S., Cho, K.Y., et al. (2006) Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. *Plant Cell Rep.* 25: 1380–1386.

Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, E. and Andersson, B. (1992) Reversible and irreversible intermediates during photoinhibition of photosystem II: stable reduced QA species promote chlorophyll triplet formation. *Proc. Natl. Acad. Sci. USA* 89: 1408–1412.

Wada, H., Gombos, Z. and Murata, N. (1994) Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress. *Proc. Natl. Acad. Sci. USA* 91: 4273–4277.

Yumoto, I., Ichihashi, D., Iwata, H., Istokovics, A., Ichise, N., Matsuyama, H., et al. (2000) Purification and characterization of a catalase from the facultatively psychrophilic bacterium *Vibrio rumoiensis* S-1(T) exhibiting high catalase activity. *J. Bacteriol.* 182: 1903–1909.

Yutthanasirikul, R., Nagano, T., Jimbo, H., Hihara, Y., Kanamori, T., Ueda, T., et al. (2016) Oxidation of a cysteine residue in elongation factor EF-Tu reversibly inhibits translation in the cyanobacterium *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* 291: 5860–5870.

Zuo, F., Yu, R., Feng, X., Khaskheli, G.B., Chen, L., Ma, H., et al. (2014) Combination of heterogeneous catalase and superoxide dismutase protects *Bifidobacterium longum* strain NCC2705 from oxidative stress. *Appl. Microbiol. Biotechnol.* 98: 7523–7534.