Zeaxanthin and Echinene Echinene Echinene Echinene Protect the Repair of Photosystem II from Inhibition by Singlet Oxygen in Synechocystis sp. PCC 6803

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(Received December 22, 2014; Accepted January 30, 2015)

Introduction

PSII, a pigment–protein complex that converts light energy to chemical energy, is particularly sensitive to inactivation by strong light, and this phenomenon is referred to as the photoinhibition of PSII (Powles 1984, Aro et al. 1993, Aro et al. 2005). In living cells, light-induced damage (photodamage) to PSII is reversed by a rapid repair system that involves the synthesis of proteins de novo (Aro et al. 1993, Aro et al. 2005). The rate of photodamage is proportional to the intensity of incident light, while the rate of repair reaches a plateau under light at relatively low intensities (Tyystjärvi and Aro 1996, Anderson and Chow 2002, Allakhverdiev and Murata 2004). Thus, photoinhibition becomes apparent under strong light when the rate of photodamage exceeds the rate of repair (Nishiyama et al. 2006). In order to understand the nature of photoinhibition, it is necessary to monitor the processes of photodamage and repair separately. Methods for the monitoring of photodamage and repair separately have been established in cyanobacteria (Gombos et al. 1994, Wada et al. 1994), green algae (Lidholm et al. 1987) and plants (Moon et al. 1995), and their application has revealed several new aspects of the mechanisms of photoinhibition (Murata et al. 2007, Nishiyama et al. 2011, Murata et al. 2012).

One of the most striking features of the newly revealed aspects of photoinhibition is the role of reactive oxygen species (ROS) in photoinhibition (Nishiyama et al. 2006). ROS are inevitably produced as by-products of photosynthetic reactions. The superoxide anion radical, hydrogen peroxide and the hydroxyl radical are generated as a result of the transport of electrons, while singlet oxygen (1O2) is generated as a result of the transfer of excitation energy (Asada 1999). The production of these ROS is stimulated under strong light (Asada 1999). In earlier studies, ROS, and in particular 1O2, were considered to be the cause of photodamage to PSII (Vass et al. 1992, Keren et al. 1997, Hideg et al. 2007). However, monitoring of photodamage and repair separately revealed that ROS act primarily
by inhibiting the repair of PSII and not by accelerating its photodamage (Nishiyama et al. 2001, Allakhverdiev and Murata 2004, Nishiyama et al. 2004). These findings led to a re-examination of the roles of the antioxidative systems that might potentially protect PSII from photoinhibition via both scavenging of ROS and depressing the production of ROS (Inoue et al. 2011, Murata et al. 2012).

Carotenoids are important components of antioxidative systems in organisms that engage in oxygenic photosynthesis. It is generally assumed that most carotenoids act by quenching the excited triplet-state Chl to prevent the production of $1O_2$ and, also, by scavenging $1O_2$ directly (Young and Frank 1996). For example, in the cyanobacterium Thermosynechococcus elongatus, 12 and 22 carotenoid molecules are located in PSII and PSI, respectively (Jordan et al. 2001, Guskov et al. 2009). Among these carotenoids, $\beta$-carotene, in particular, is essential for oxygenic photosynthesis. For example, a mutant of the cyanobacterium Synechocystis sp. PCC 6803 (hereafter Synechocystis) that is deficient in $\beta$-carotene is unable to form a functional PSII and is unable to grow photoautotrophically (Sozer et al. 2010). Xanthophylls, such as zeaxanthin and echinenone, are also important for the protection of the photosynthetic machinery from excess light. In mutants of Synechocystis deficient in either zeaxanthin or echinenone, net photosynthetic activity was more sensitive to strong light than that in wild-type cells, while a double mutant deficient in both zeaxanthin and echinenone exhibited more pronounced sensitivity than the individual single mutants (Schafer et al. 2005). A similar mutant of Synechocystis, which lacked almost all xanthophylls, accumulated ROS and reactive nitrogen species at high levels (Zhu et al. 2010). However, it remains to be determined whether carotenoids protect PSII from photodamage or protect the repair of PSII.

Some carotenoids act by dissipating excitation energy as heat, and we refer to this process herein as thermal dissipation. In Synechocystis, 3’-hydroxyechinenone, which is bound to the so-called orange carotenoid protein (OCP), is responsible for thermal dissipation, which can be monitored as a major component of non-photochemical quenching (NPQ). Under strong light, OCP binds to phycobilisomes and suppresses the transfer of excitation energy from phycobilisomes to reaction centers, dissipating part of the energy as heat (El Bissati et al. 2000, Kerfeld et al. 2003, Wilson et al. 2006, Wilson et al. 2008). Thus, the role of carotenoid-mediated thermal dissipation in photoinhibition also merits re-examination.

In the present study, we examined the roles of carotenoids in the photoinhibition of PSII using mutants of Synechocystis that were deficient in either or both zeaxanthin and echinenone, as well as a mutant deficient in OCP. All the various mutant cells were more sensitive to photoinhibition than were wild-type cells, and all exhibited impaired ability to repair PSII in the absence of any change in the rate of photodamage. The impaired repair was associated with decreased rates of synthesis of the D1 protein and increased levels of production of $1O_2$. Thus, it appears that carotenoids protect the repair of PSII by scavenging $1O_2$ and also by mediating the thermal dissipation of excitation energy, both of which lead to decreases in intracellular levels of $1O_2$.

**Results**

**Generation of mutants**

Fig. 1 shows part of the biosynthetic pathway for the synthesis of carotenoids in Synechocystis (Takaichi and Mochimaru 2007). The major carotenoids in this organism are $\beta$-carotene, myxoxanthophyll, zeaxanthin, and 3’-hydroxyechinenone. Myxoxanthophyll is synthesized from $\gamma$-carotene in reactions catalyzed by several enzymes, which include CrtR.

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zeaxanthin, echinenone, 3′-hydroxyechinenone, myxoxanthophyll (myxo 2′-dimethyl-fucoside) and synechoxanthin (Takaichi et al. 2001, Graham et al. 2008). Zeaxanthin and echinenone are synthesized from β-carotene in reactions catalyzed by β-carotene hydroxylase (CrtR) and β-carotene ketolase (CrtO), respectively, and it seems likely that myxoxanthophyll is synthesized from γ-carotene by several enzymes, including CrtR (Takaichi and Mochimaru 2007). 3′-Hydroxyechinenone is synthesized either from echinenone by CrtR or from β-cryptoxanthin, a precursor to zeaxanthin, by CrtO (Takaichi and Mochimaru 2007). Synechoxanthin, a carotenoid that was recently identified, might be synthesized from β-carotene (Graham et al. 2008).

We generated a mutant deficient in zeaxanthin by insertional mutagenesis of the \( \text{crtR} \) gene; a mutant deficient in echinenone by insertional mutagenesis of the \( \text{crtO} \) gene; and a mutant deficient in both zeaxanthin and echinenone by insertional mutagenesis of both the \( \text{crtR} \) and \( \text{crtO} \) genes (Fig. 2A). To examine the role of carotenoid-mediated thermal dissipation in photoinhibition, we also generated a mutant deficient in OCP by insertional mutagenesis of the \( \text{ocp} \) gene (Fig. 2A). The complete segregation of each genome was confirmed by PCR with forward and reverse primers and the appropriate genomic DNA as template (Fig. 2B).

**Levels of carotenoids in mutant cells**

We analyzed the levels of various carotenoids in wild-type and mutant cells by HPLC. There was no detectable zeaxanthin in \( \text{crtR} \) mutant cells and no detectable echinenone in \( \text{crtO} \) mutant cells. Neither zeaxanthin nor echinenone was detected in \( \text{crtRcrtO} \) double-mutant cells (Fig. 3). In contrast, the amounts of β-carotene in the three lines of mutant cells were higher than that in wild-type cells. Myxoxanthophyll was detected in wild-type and \( \text{crtO} \) cells but not in \( \text{crtR} \) and \( \text{crtRcrtO} \) cells, and...
deoxymyxoxanthophyll (deoxymyroxyl 2'-dimethyl-fucoside), a precursor to myxoxanthophyll, was detected in \( \text{crtR} \) and \( \text{crtRcrtO} \) cells but not in wild-type and \( \text{crtO} \) cells. \( \beta \)-Cryptoxanthin was detected at low levels in wild-type cells, but it was undetectable in the mutant cells. Synechoxanthin was detected at similar levels in all lines of cells examined. We were unable to detect \( 3'0 \)-hydroxyechinenone, probably due to its low abundance.

Absence of zeaxanthin and echinenone enhances the extent of photoinhibition of PSII without affecting its rate of photodamage

When cells were exposed to light at 1,500 \( \mu \)mol photons \( m^{-2} s^{-1} \) at 25°C, the activity of PSII in the \( \text{crtR} \) and \( \text{crtO} \) cells declined more rapidly than that in wild-type cells (Fig. 4A). The activity of PSII in \( \text{crtRcrtO} \) cells declined much more rapidly than in the other three lines. After illumination for 180 min, the activity of PSII in \( \text{crtRcrtO} \) cells fell to approximately 50% of that in wild-type cells. Thus, the absence of either zeaxanthin or echinenone increased the extent of photoinhibition of PSII, and the absence of both carotenoids together increased the extent of photoinhibition even further. However, when cells were exposed to light at the same intensity in the presence of chloramphenicol, which blocks the repair of PSII, the activity of PSII in all lines of mutant cells declined at the same rate as that in wild-type cells (Fig. 4B). Thus, it appeared that the absence of zeaxanthin and echinenone might not accelerate photodamage to PSII but might, rather, inhibit the concurrent repair of photodamaged PSII.

Absence of zeaxanthin and echinenone suppresses the synthesis of the D1 protein de novo

The synthesis of the D1 protein de novo plays a central role in the repair of photodamaged PSII (Aro et al. 1993, Aro et al. 2005). To examine the effects of the absence of the carotenoids of interest on the synthesis de novo of the D1 protein, we monitored the incorporation of \( ^{35}S \)-labeled methionine plus cysteine into proteins during the exposure of cells to strong light at 1,500 \( \mu \)mol photons \( m^{-2} s^{-1} \). Fig. 5A shows the patterns of pulse-labeled proteins from thylakoid membranes of wild-type and double-mutant cells after SDS–PAGE and the time courses of the synthesis of the labeled D1 protein. The rate of synthesis of the D1 protein de novo in \( \text{crtRcrtO} \) cells was approximately 20% lower than that in wild-type cells (Fig. 5B). Thus, the absence of zeaxanthin and echinenone appeared to suppress the synthesis of the D1 protein de novo during photoinhibition.

The patterns of pulse-labeled proteins revealed that not only the synthesis of the D1 protein but also that of almost all the proteins of thylakoid membranes was suppressed in the \( \text{crtRcrtO} \) cells (Fig. 5A). We examined the global effects of the absence of zeaxanthin and echinenone on the synthesis de novo of cellular proteins by quantifying pulse-labeled proteins by liquid scintillation counting. Levels of newly synthesized proteins in thylakoid membranes from \( \text{crtRcrtO} \) cells were lower than those from wild-type cells (Fig. 5C). Thus, the absence of zeaxanthin and echinenone had a negative effect on the global synthesis of proteins during strong illumination.

Absence of OCP does not affect photodamage to PSII but suppresses its repair

In \textit{Synechocystis}, echinenone plays a dual role in photoprotection: it acts as an antioxidant, and its derivative, \( 3'0 \)-hydroxyechinenone, a cofactor of OCP, is responsible for the thermal dissipation of excitation energy (Wilson et al. 2006). To dissect this dual role in photoinhibition, we generated a mutant deficient in OCP (Fig. 2). Then we investigated the thermal dissipation of excitation energy by monitoring levels of NPQ, which is induced mainly by thermal dissipation, and the transition between states of PSI and PSII. Levels of NPQ in wild-type cells rose as the intensity of actinic light was increased (Fig. 6A), a result that confirmed previously reported
light-dependent changes in levels of NPQ in cyanobacteria (Sonoike et al. 2001). However, increases in the levels of NPQ in ocp and crtRcrtO cells were suppressed, to a similar extent, when the intensity of light was >500 μmol photons m⁻² s⁻¹ (Fig. 6A). In our analysis of the state transitions of the photosystems, we found that illumination of dark-adapted wild-type cells with weak light up to 100 μmol photons m⁻² s⁻¹ changed the state from state 2 to state 1, suggesting that a large fraction of phycobilisomes might have migrated from PSI to PSII (Fig. 6B). Further illumination with stronger light reversed this phenomenon, with a transition from state 1 to state 2, and this observation suggested that a large fraction of phycobilisomes might have migrated back to PSI. However, the light-dependent transitions between states were the same in wild-type and ocp cells. Thus, suppression of increases in levels of NPQ might have been due solely to impairment of thermal dissipation, and the induction of NPQ that depends on state transition might have occurred in the absence of OCP. In other words, thermal dissipation via OCP might be independent of the state transition.

We then compared mutant and wild-type lines in terms of the extent of photoinhibition of PSII. When cells were exposed to light at 1,500 μmol photons m⁻² s⁻¹ at 25°C, the activity of PSII in ocp cells declined more rapidly than that in wild-type cells (Fig. 4A). The extent of photoinhibition in ocp cells was similar to that in crtR and in ocp cells but smaller than that in the crtRcrtO cells. However, when cells were exposed to light at 1,500 μmol photons m⁻² s⁻¹ at 25°C in the presence of chloramphenicol, the activity of PSII in ocp cells declined at the same rate as that in wild-type cells (Fig. 4B). Thus, it appeared that the absence of thermal dissipation due to the lack of OCP might have enhanced the extent of photoinhibition of PSII by inhibiting the repair of PSII rather than by affecting its photodamage.

**Impaired thermal dissipation does not affect the rate of electron transport**

The effects of a defect in thermal dissipation led us to postulate that the rate of electron transport might be accelerated under strong light. To examine this possibility, we measured the effective quantum yield of PSII (ΦPSII), together with the yields of the regulated dissipation of energy (ΦNPQ) and the non-regulated dissipation of energy (ΦED) under blue actinic light at 800 μmol photons m⁻² s⁻¹. Compared with wild-type cells, all lines of mutant cells examined gave lower values of ΦNPQ but higher values of ΦED (Fig. 7A). In contrast, values of ΦPSII were almost identical for all lines examined. These observations suggested that impaired thermal dissipation might not affect the rate of electron transport.

Next, we measured the activity of the whole-chain transport of electrons from water to methyl viologen under light at various intensities. The electron transport activity did not differ between wild-type and ocp cells (Fig. 7B), confirming our hypothesis that impairment of thermal dissipation might not affect the rate of electron transport.

**Impaired thermal dissipation and the absence of zeaxanthin and of equinenone accelerate the production of 1O₂**

The elevated value of ΦED in ocp cells led us to postulate that the energy that would otherwise have been dissipated as heat might have been transferred to molecular oxygen to yield 1O₂. We measured the rate of production of 1O₂ from cells under strong illumination at 2,500 μmol photons m⁻² s⁻¹ for 5 min by monitoring the light-induced uptake of O₂ in the presence of histidine. The rate of uptake of O₂ in ocp cells was higher than that in wild-type cells (Fig. 8), indicating that 1O₂ was produced more abundantly in ocp cells. Addition of DCMU, which blocks the transport of electrons, did not affect the uptake of O₂ to
any significant extent. It is possible that the energy used for electron transport might have been dissipated as fluorescence. However, inclusion of NaN₃, a quencher of ¹O₂, depressed the uptake of O₂ to similar low levels in both wild-type and ocp cells, confirming the accurate detection of ¹O₂. These observations suggested that the impairment of thermal dissipation might have accelerated the production of ¹O₂. In other words, the energy to be dissipated as heat might have been transferred to molecular oxygen to yield ¹O₂. In crtRcrtO cells, the light-induced uptake of O₂ was further enhanced, indicating that the absence of both zeaxanthin and echinenone results in even higher rates of production of ¹O₂.

**Discussion**

**Roles of zeaxanthin and echinenone in the protection of PSII from photoinhibition**

Enhanced photoinhibition in the absence of zeaxanthin and echinenone has been reported previously (Schäfer et al., 2005), but our goal was to determine whether these carotenoids protect PSII from photodamage or, alternatively, whether they protect the repair of PSII. We found that the absence of these carotenoids did not accelerate photodamage to PSII but inhibited its repair, demonstrating that these carotenoids act exclusively by protecting the repair of PSII. Furthermore, the accelerated production of ¹O₂ that we observed in the double mutant that was deficient in both zeaxanthin and echinenone suggests that these carotenoids might protect the repair of PSII from inhibition by ¹O₂. The action of the carotenoids in the repair process resembled that of α-tocopherol, an antioxidant that scavenges ¹O₂ efficiently. In *Synechocystis*, in the absence of α-tocopherol, the repair of PSII is inhibited but there is no effect on photodamage to PSII (Inoue et al., 2011). Thus, it seems likely that protective compounds that are capable of scavenging ¹O₂ or depressing its accumulation might, in general, act by protecting the repair of PSII during photoinhibition (Nishiyama and Murata, 2014).

In mutant cells deficient in zeaxanthin and/or echinenone, levels of β-carotene and deoxymyxoxanthophyll increased...
Fig. 3). Nonetheless, the sensitivity to photoinhibition of these mutant cells was higher than that of wild-type cells, indicating that elevated accumulation of $b$-carotene and deoxymyxoxanthophyll cannot compensate for the lack of zeaxanthin or echinenone. It seems likely, therefore, that zeaxanthin and echinenone play specific roles in the protection of the repair of PSII.

**Role of $^1O_2$ in the photoinhibition of PSII**

Several studies have suggested that $^1O_2$ might be a cause of photodamage to PSII. For example, in the ‘acceptor-side’ and ‘low-light’ models, it has been proposed that charge recombination in the reaction center might trigger the formation of excited triplet-state Chl, which is able to transfer excitation energy to molecular oxygen to generate $^1O_2$ that attacks the D1 protein directly (Vass et al. 1992, Keren et al. 1997). In contrast, the more recent ‘two-step’ model suggests that photodamage might occur in two steps: in the first step, the oxygen-evolving complex and, most probably, the manganese cluster are damaged upon absorption of UV and blue light; in the second step, the reaction center is damaged by visible light that is absorbed by Chl (Ohnishi et al. 2005, Hakala et al. 2005). According to the ‘two-step’ model, ROS are unlikely to be the primary cause of photodamage and, in particular, of damage at the first step. The ‘two-step’ model is supported by observations of the actions of ROS (Nishiyama et al. 2011, Murata et al.
For example, increases in intracellular levels of $^1$O$_2$ in *Synechocystis* by photosensitizers, such as rose bengal and ethyl eosin, did not accelerate photodamage to PSII but inhibited the repair of PSII (Nishiyama et al. 2001, Nishiyama et al. 2004). As mentioned above, the absence of $\alpha$-tocopherol in *Synechocystis* did not accelerate photodamage to PSII but inhibited its repair (Inoue et al. 2011). In addition, our present observations with carotenoid-deficient mutants reinforce the hypothesis that $^1$O$_2$ might act primarily by inhibiting the repair of PSII. However, it should be noted here that the mechanism of photodamage remains a matter of dispute (Vass 2012, Tyystjärvi 2013). The earlier $^1$O$_2$-dependent mechanism was supported by studies with *Synechocystis* in which photodamage was affected (i) by mutations in the D1 protein that altered the redox potential of pheophytin (Rehman et al. 2013); (ii) by overexpression of flavodiiron proteins Flv2 and Flv4 (Bersanini et al. 2014); and (iii) by knockout of the genes for three sigma factors, SigC, SigD and SigE, which resulted in marked changes in the relative levels of various carotenoids (Hakkila et al. 2014).

Mechanism for the protection of the repair of PSII by zeaxanthin and echinenone

The absence of both zeaxanthin and echinenone resulted in the suppression of the synthesis de novo of proteins, such as the D1 protein, under strong light. This observation suggests that these carotenoids might protect the synthesis de novo of the D1 protein from suppression by $^1$O$_2$, thereby, to a large extent, contributing to the enhancement of the repair of PSII during photoinhibition. This hypothesis is consistent with the previous finding that the intracellular production of $^1$O$_2$ in response to photosensitizers inhibits the synthesis of the D1 protein at the elongation step of translation under strong light (Nishiyama et al. 2004). The global suppression of protein synthesis in the absence of zeaxanthin and echinenone suggests that $^1$O$_2$ might inactivate the protein-synthetic system directly. An earlier study, using a translation system in vitro derived from *Synechocystis*, demonstrated that H$_2$O$_2$ inactivates the translational machinery via oxidation of EF-G, a translation factor essential for the elongation of peptides, with resultant inhibition of the synthesis of the D1 protein (Kojima et al. 2007). The oxidation of EF-G by H$_2$O$_2$ leads to formation of an intramolecular disulfide bond between specific cysteine residues (Kojima et al. 2009). The particular sensitivity of EF-G to oxidation is also evident in the case of the translational machinery in *Escherichia coli* (Nagano et al. 2012). It remains to be determined whether zeaxanthin and echinenone protect EF-G from oxidation by $^1$O$_2$.

Role of thermal dissipation in the protection of PSII from photoinhibition

The effect of the absence of OCP on photoinhibition of PSII suggests that the thermal dissipation of excitation energy might act by protecting the repair of PSII rather than by protecting PSII from photodamage. Although it was reported that red-light-induced photodamage to PSII, as monitored in the presence of lincomycin, was affected by the absence or overexpression of OCP in *Synechocystis* (Sedoud et al. 2014), our hypothesis is supported by the effect of the impairment of thermal dissipation on photoinhibition in Arabidopsis. The npq1 and npq4 mutations in Arabidopsis had a much greater negative effect on the repair of PSII than on photodamage to PSII (Sarvikas et al. 2006, Takahashi et al. 2009). It seems that thermal dissipation acts similarly in photoinhibition in *Synechocystis* and Arabidopsis, although the mechanism of thermal dissipation differs considerably in the two organisms. The thermal dissipation of excitation energy involves OCP and 3’-hydroxyechinenone in *Synechocystis* (Wilson et al. 2006),
whereas it involves PsbS and the xanthophyll cycle in Arabidopsis (Bugos and Yamamoto 1996, Li et al. 2000). Moreover, in Synechocystis, the absence of OCP did not affect the rate of electron transport but stimulated the production of 1O2 under strong light (Figs. 7, 8). These observations suggest that the dissipation of excitation via OCP might not decelerate the rate of electron transport to prevent the production of ROS on the acceptor side of PSI but might, rather, suppress the formation of 1O2 in the antenna of PSI. The independence of thermal dissipation from the transport of electrons was also observed in npq1 and npq4 mutants of Arabidopsis (Takahashi et al. 2009), and the stimulated production of 1O2 in the absence of OCP has been reported previously in Synechocystis (Sedoud et al. 2014). 3'-Hydroxyechinenone, a derivative of echinenone, is normally responsible for thermal dissipation within OCP and is interchangeable with echinenone (Punginelli et al. 2009). Thus, echinenone might depress levels of 1O2 not only by scavenging 1O2 either directly or via quenching of the triplet-state Chl but also by the dissipation as heat of excitation energy stored in 3'-hydroxyechinenone.

**Conclusion**

The presence of zeaxanthin and echinenone reduces the extent of photoinhibition of PSII by protecting the repair of PSII and not by protecting PSII from photodamage. These carotenoids act by depressing the levels of 1O2 via scavenging of 1O2, and the thermal dissipation of excitation energy to protect, from oxidative damage by 1O2, the synthesis de novo of proteins that are required for the repair of PSII.

**Materials and Methods**

**Cells and culture conditions**

Cells of a glucose-tolerant strain (hereafter referred to as the wild type) and of mutant strains of Synechocystis sp. PCC 6803 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) CO2, as described previously (Inoue et al. 2011). Cells in cultures with an optical density at 730 nm of 1.0±0.1 were used for assays (unless otherwise noted).

**Generation of mutants**

The upstream and downstream regions of the crtR (slr1668), crtO (slr0088) (Takahashi and Mochimaru 2007) and ocp (slr1663) (Punginelli et al. 2009) genes were amplified from the genomic DNA of Synechocystis by PCR with appropriate forward and reverse primers that included a BamHI site for crtR and crtO and an Nhel site for ocp (sequences of all primers are available on request). The amplified DNA fragments were cloned into the pGEM®-T Easy vector (Promega). A streptomycin/spectinomycin resistance cassette was inserted into the BamHI site of crtR, and a kanamycin resistance cassette was inserted into the BamHI site of crtO and the Nhel site of ocp. The resultant plasmids were used to transform wild-type Synechocystis by homologous recombination.

**Analysis and identification of carotenoids**

Pigments were extracted from wild-type and mutant cells with a mixture of acetone and methanol (7:2, v/v) and were analyzed by HPLC on a system equipped with a μBondapack C18 column (8 mm × 100 mm; RCM type; Waters). Carotenoids were eluted with a mixture of methanol and water (9:1, v/v) for 20 min and then with 100% methanol, at a rate of 1.8 ml min−1 (Mochimaru et al. 2008). Zeaxanthin and β-carotene were detected at 450 nm, while echinenone, myxoxanthophyll and synechocyanin were detected at 475 nm. For quantification, the molar extinction coefficients in the eluent were assumed to be the same, as described previously (Takaichi et al. 2001).

**Assay of the photoinhibition of PSII**

Cells were exposed to light at 1,500 μmol photons m−2 s−1 at 25°C for designated periods of time to induce the photoinhibition of PSII. For assays of photodamage, chloramphenicol was added to the suspension of cells at a final concentration of 200 μg ml−1 just before the onset of illumination. The activity of PSII was measured at 25°C in terms of the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone and 1 mM K3Fe(CN)6 with a Clark-type oxygen electrode (Hansatech Instruments). The activity of whole-chain electron transport was measured at 25°C in terms of the uptake of oxygen in the presence of 1 mM methyl viologen and 1 mM KCN.

**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 35S-labeled methionine plus cysteine (EasyTag™ EXPRE35S™, PerkinElmer), as described previously (Nishiyama et al. 2004). Aliquots of 7 ml of each were withdrawn at 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 membrane proteins were separated by SDS-PAGE on a 15% polyacrylamide gel that contained 6 M urea. Labeled proteins on the gel were visualized with an imaging analyzer (FLA-7000; Fujifilm) and levels of the D1 protein were determined densitometrically. Levels of labeled proteins in thylakoid membranes were also quantitated by liquid scintillation counting, as described previously (Kojima et al. 2007).

**Measurements of Chl fluorescence**

The fluorescence of Chl was measured with a pulse-amplitude fluorimeter (PAM-2000; Waltz). Cells were placed in darkness for 5 min prior to measurements. Minimal fluorescence (F0), fluorescence under steady-state conditions (Fm), the maximum fluorescence of light-acclimated cells (Fm′) and maximum fluorescence, as determined in the presence of DCMU (Fm″), were used for calculations of the parameters NPQ, ΦPSII, ΦPSO and ΦLD as follows: NPQ = (Fm″ − Fm)/Fm″; ΦPSII = (Fm′ − Fm)/Fm′; ΦPSO = Fm″ − Fm′ and ΦLD = Fm/Fm′ (Ogawa et al. 2013). For determination of NPQ, a 0.8 s flash of saturating light was applied for determination of Fm″ after illumination by white actinic light at designated intensities for 4 min. For determination of ΦPSII, ΦPSO and ΦLD ≥ 0.8 s flash of saturating light was applied for measurement of Fm″ after illumination by blue actinic light through a blue optical filter (B-460; Hoya) at 800 μmol photons m−2 s−1 for 5 min. Fm was measured in the presence of 10 μM DCMU with illumination by actinic light.

**Fluorescence emission spectra**

Fluorescence emission spectra were recorded at 77K with a fluorescence spectrometer (FP-8500; JASCO) equipped with a low-temperature attachment (PL-830; JASCO), as described elsewhere (Ogawa et al. 2013). Cells were either incubated in darkness for 10 min or illuminated at the designated intensities for 5 min. The cells were then exposed to light at 625 nm with a slit width of 10 nm for excitation of phycocyanin. The fluorescence spectra were recorded with a fluorescence slit width of 2.5 nm and a resolution of 0.2 nm. The spectra were recorded five times for each sample, and averages of data from three independent cultures were determined. The spectra were corrected for the sensitivity of the photomultiplier and the spectrum of the light source by use of a secondary standard light source (ESC-642; JASCO).

**Detection of 1O2**

The production of 1O2 in intact cells was detected by measuring the rate of the light-induced uptake of oxygen in the presence of histidine, as described previously (Rehman et al. 2013). Cells in cultures with an optical density at 730 nm...
of 0.5 ± 0.1 were exposed to light at 2,500 μmol photons m⁻² s⁻¹ at 25°C in the presence of 5 mM histidine and in its absence, and the evolution of oxygen was measured in the absence of electron acceptors. The generation of O₂ was quantitated by subtracting the rate of the evolution of oxygen in the absence of electron acceptors. The generation of 1O₂ was measured in the absence of 5 mM histidine and in its absence, and the evolution of oxygen was also performed in the presence of 10 μM DCMU and 10 mM NaN₃.

**Funding**

This work was supported by the Japan Society for the Promotion of Science [Grants-in-Aid for Scientific Research (Nos. 24570039 and 25119704 to Y.N.)].

**Acknowledgments**

The authors thank Mr. Kenta Suzuki (Waseda University) for state transition analysis, and Mr. Tomohisa Niimi (Saitama University) for radioisotope analysis.

**Disclosures**

The authors have no conflicts of interest to declare.

**References**


