How Does Cyclic Electron Flow Alleviate Photoinhibition in Arabidopsis?\textsuperscript{1}[W][OA]

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Cyclic electron flow (CEF) around photosystem I has a role in avoiding photoinhibition of photosystem II (PSII), which occurs under conditions in which the rate of photodamage to PSII exceeds the rate of its repair. However, the molecular mechanism underlying how CEF contributes to photoprotection is not yet well understood. We examined the effect of impairment of CEF and thermal energy dissipation (qE) on photoinhibition using CEF (\textit{pgr5}) and qE (\textit{npq1} and \textit{npq4}) mutants of Arabidopsis (\textit{Arabidopsis thaliana}) exposed to strong light. Impairment of CEF by mutation of \textit{pgr5} suppressed qE and accelerated photoinhibition. We found that impairment of qE, by mutations of \textit{pgr5}, \textit{npq1}, and \textit{npq4}, caused inhibition of the repair of photodamaged PSII at the step of the de novo synthesis of the D1 protein. In the presence of the chloroplast protein synthesis inhibitor chloramphenicol, impairment of CEF, but not impairment of qE, accelerated photoinhibition, and a similar effect was obtained when leaves were infiltrated with the protonophore nigericin. These results suggest that CEF-dependent generation of $\Delta$PH across the thylakoid membrane helps to alleviate photoinhibition by at least two different photoprotection mechanisms: one is linked to qE generation and prevents the inhibition of the repair of photodamaged PSII at the step of protein synthesis, and the other is independent of qE and suppresses photodamage to PSII.

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PSII is rapidly and effectively repaired through the replacement of photodamaged PSII proteins with newly synthesized proteins, primarily the D1 protein (Ohad et al., 1984; Mattoo and Edelman, 1987; Aro et al., 1993b, 2005). Thus, photoinhibition occurs only under conditions in which the rate of photodamage exceeds the rate of its repair (Aro et al., 1993b; Murata et al., 2007; Takahashi and Murata, 2008). To prevent photoinhibition, photoprotective mechanisms are used by the plant to both suppress the photodamage to PSII (e.g. chloroplast avoidance movement [Kasahara et al., 2002]) and minimize oxidative inhibition of the repair of photodamaged PSII (e.g. photorespiratory pathway and Calvin cycle [Takahashi et al., 2007] and reactive oxygen species-scavenging systems [Nishiyama et al., 2001]).

Under conditions of excess light, plants dissipate unused absorbed light energy harmlessly as heat in the antenna proteins of PSII (for review, see Niyogi, 1999). This mechanism is called energy-dependent thermal dissipation (qE) and is measured as a component of nonphotochemical quenching of chlorophyll fluorescence (NPQ). NPQ can be subdivided into three components: qE, photoinhibition (qI), and state transitions (qT). Under strong light conditions, qE is the major component of NPQ and quenches up to 80% of the excited chlorophyll in plants. qE is associated with the conversion of violaxanthin to zeaxanthin, via the intermediate antheraxanthin, by the catalyst violaxanthin deepoxidase (VDE; Niyogi et al., 1997, 1998) and conversion of violaxanthin to zeaxanthin, via the intermediate antheraxanthin, by the catalyst violaxanthin deepoxidase (VDE; Niyogi et al., 1997, 1998) and protonation of PsbS (Li et al., 2002), which is an integral membrane subunit of PSII. Both reactions are regulated by low lumenal pH, which is accompanied by the generation of ΔpH (Munekage et al., 2002; Shikanai, 2007). Thus, generation of increased ΔpH through CEF is important for the activation of qE. Impairment of qE by the mutation of genes coding for the proteins VDE (npq1) and PsbS (npq4) in Arabidopsis (Arabidopsis thaliana) causes acceleration of photoinhibition of PSII under strong light (Niyogi et al., 1998; Li et al., 2002). Since, in the previous photoinhibition model, photodamage to PSII was proposed to be attributable to light absorbed by photosynthetic pigments, energy dissipation through qE was assumed to help avoid photodamage to PSII. However, recent studies have demonstrated that impairment of qE had no significant effect on the process of photodamage per se to PSII, which is consistent with the new photoinhibition model (Nishiyama et al., 2006; Sarvikas et al., 2006). Therefore, mechanisms of photoprotection associated with CEF and qE are still undefined.

To further understand the role of CEF in photoprotection, we examined the effect of impairment of CEF and qE on photoinhibition of PSII under strong light using Arabidopsis mutants impaired in CEF (pgr5) and qE development (npq1 and npq4). The results clearly suggest that reduction in both CEF and qE resulted in inhibition of the synthesis of the D1 protein under strong light, but only reduction in CEF caused an increase in direct photodamage to PSII.

RESULTS

Characteristics of the pgr5 Mutant

All plants used in this study were grown in medium light at 100 μmol photons m⁻² s⁻¹. The pgr5 mutant, but not the npq1 or the npq4 mutant, grew slightly slower than the wild type. However, there was no significant difference in phenotype of mature leaves between the wild type and all mutants used. The photosynthetic CO₂ fixation rate in mature leaves of the wild type was 6.2, 17.0, and 18.5 μmol CO₂ m⁻² s⁻¹ in light at 100, 500, and 1,000 μmol photons m⁻² s⁻¹, respectively (Fig. 1A). The CO₂ fixation rates were suppressed by 10% to 20% in the pgr5 mutants at all light intensities (Fig. 1A). The photosynthetic O₂ evolution rate in the wild type was 6.7, 18.4, and 21.1 μmol O₂ m⁻² s⁻¹ in light at 100, 500, and 1,000 μmol photons m⁻² s⁻¹, respectively (Fig. 1B). The effect of mutation of pgr5 on the O₂ evolution rate was similar to that on the CO₂ fixation rate (Fig. 1B). The CO₂ fixation rate and the O₂ evolution rate were suppressed by 10% to 20% in the npq4 mutant, but not significantly in the npq1 mutant, at any light intensity tested (Fig. 1). These results indicate that there was no significant difference in the effect of mutations of pgr5, npq1, and npq4...
on the photosynthetic activity at all light intensities, suggesting that reductions of CEF and qE had no direct effect on the photosynthetic activity.

Impairment of CEF Accelerated Photoinhibition in Both the Presence and Absence of Chloramphenicol

The effect of impairment of CEF by the mutation of pgr5 on qE was examined by the measurement of NPQ (Fig. 2A). When the wild type was exposed to light at 1,000 μmol photons m$^{-2}$ s$^{-1}$ for 10 min, the level of NPQ was increased to 2.5. However, in the pgr5 mutant, the level of NPQ increased to only 40% of the wild-type level. When leaf discs from the wild type and the pgr5 mutant were infiltrated with 250 μM antimycin A, which inhibits PGR5-dependent CEF, the level of NPQ was strongly suppressed in both the wild type and the pgr5 mutant, and the effect of pgr5 mutation on the level of NPQ was almost abolished (Supplemental Fig. S1A).

To examine the effect of impairment of CEF on photoinhibition of PSII, we measured the maximal quantum yield of PSII ($F_{v}/F_{m}$) in the wild type and the pgr5 mutant after exposure to strong light at 1,000 μmol photons m$^{-2}$ s$^{-1}$ (Fig. 2B). In the wild type, the $F_{v}/F_{m}$ declined to 60% of the initial level after exposure for 3 h. However, in the pgr5 mutant, the $F_{v}/F_{m}$ declined to 40% of the initial level. The presence of antimycin A accelerated the decrease in $F_{v}/F_{m}$ in both the wild type and the pgr5 mutant and completely abolished the effect of pgr5 mutation on the decrease in the $F_{v}/F_{m}$ (Supplemental Fig. S1B). These results indicate that impairment of the CEF causes acceleration of photoinhibition. Antimycin A accelerated photoinhibition in the pgr5 mutant, suggesting that acceleration of photoinhibition caused by antimycin A was not only due to inhibition of antimycin A (PGR5)-dependent CEF but also inhibition of other reactions (i.e. the alternative pathway in mitochondrial respiration).

The effect of impairment of CEF on the process of photodamage to PSII was examined by the measurement of the $F_{v}/F_{m}$ after exposure to strong light in the presence of chloramphenicol (Fig. 2B). Leaf discs from the wild type and the pgr5 mutant were vacuum infiltrated with chloramphenicol and then exposed to strong light at 1,000 μmol photons m$^{-2}$ s$^{-1}$ for 3 h. Chloramphenicol accelerated the decrease in the level of $F_{v}/F_{m}$ in both the wild type and the pgr5 mutant, and the extent of decrease was significantly faster in the pgr5 mutant (Fig. 2B). However, the effect of pgr5 mutation on the decrease in the level of $F_{v}/F_{m}$ was completely abolished in the presence of antimycin A (Supplemental Fig. S1). These results indicate that impairment of CEF caused acceleration of the photodamage to PSII.

Impairment of qE Accelerated Photoinhibition Only in the Absence of Chloramphenicol

To examine whether the acceleration of photoinhibition caused by impairment of CEF is attributable to impairment of qE, the effect of mutations of npq1 and npq4 on a decrease in the level of the $F_{v}/F_{m}$ was measured in the absence or presence of chloramphenicol. When npq1 and npq4 mutants were exposed to strong light at 1,000 μmol photons m$^{-2}$ s$^{-1}$ for 10 min, the level of NPQ was induced to 40% of the wild type level in both mutants and was indistinguishable from that in the pgr5 mutant (Fig. 2A). When leaf discs were exposed to strong light in the absence of chloramphenicol, mutations of npq1 and npq4 caused acceleration of a decrease in the level of the $F_{v}/F_{m}$ (Fig. 2B). However, in the presence of chloramphenicol, there was no significant effect of npq1 and npq4 mutations on the decrease in the level of the $F_{v}/F_{m}$ (Fig. 2B). These results indicate that impairment of qE by mutations of npq1 and npq4 caused acceleration of photoinhibition through inhibition of the repair of photodamaged PSII.
but not acceleration of the photodamage to PSII. Thus, acceleration of photodamage due to impairment of CEF by the mutation of \textit{pgr5} was not attributable to suppression of qE.

**Methyl Viologen Accelerated Photoinhibition Only in the Absence of Chloramphenicol**

We examined whether the production of hydrogen peroxide caused acceleration of photoinhibition of PSII in Arabidopsis. Leaf discs from the wild type and the \textit{pgr5} mutant were vacuum infiltrated with 200 \(\mu\)M methyl viologen, which generates superoxide and hydrogen peroxide through the electron transfer to oxygen at PSI. In the absence of chloramphenicol, methyl viologen accelerated a decrease in the level of \(F_v/F_m\) in both the wild type (Fig. 3B) and the \textit{pgr5} mutant (Supplemental Fig. S2B). However, in the presence of chloramphenicol, there was no significant effect of methyl viologen on the decrease in the level of \(F_v/F_m\) in both the wild type (Fig. 3B) and the \textit{pgr5} mutant (Supplemental Fig. S2B). These results indicate that the production of reactive oxygen species, such as hydrogen peroxide and superoxide, accelerated photoinhibition through inhibition of the repair of photodamaged PSII but not acceleration of the photodamage to PSII. These results were consistent with a previous report in cyanobacteria (Nishiyama et al., 2001). Thus, acceleration of photodamage caused by impairment of CEF is not attributable to the production of reactive oxygen species.

**Nigericin Accelerated Photoinhibition in Both the Presence and Absence of Chloramphenicol**

We examined whether inhibition of the generation of \(\Delta\text{pH}\) across the thylakoid membrane causes acceleration of photoinhibition of PSII using the protonophore nigericin. Leaf discs from the wild type and the \textit{pgr5} mutant were vacuum infiltrated with 1 mM nigericin. The effect of nigericin as a protonophore was examined by suppression of the NPQ development under strong light in both the wild type (Fig. 3A) and the \textit{pgr5} mutant (Supplemental Fig. S2A). When leaf discs from the wild type and the \textit{pgr5} mutant were exposed to strong light, nigericin accelerated the decrease in the level of \(F_v/F_m\) in the presence and absence of chloramphenicol in both plants (Fig. 3B; Supplemental Fig. S2B). These results indicate that nigericin increased photoinhibition at least partially through acceleration of the photodamage to PSII, indicating that impairment of the generation of \(\Delta\text{pH}\) across the thylakoid membrane causes acceleration of the photodamage to PSII.

**Reduction of Both CEF and qE Inhibited the de Novo Synthesis of the D1 Protein**

To examine whether impairments of the CEF and qE causes inhibition of the de novo synthesis of the D1 protein, we investigated the uptake of \[^{35}\text{S}]\text{Met/Cys}\) into newly synthesized proteins of thylakoid membranes in the wild type and \textit{pgr5}, \textit{npq1}, and \textit{npq4} mutants. After leaf discs were vacuum infiltrated with \[^{35}\text{S}]\text{Met/Cys}\), they were incubated in low light at 100 \(\mu\)mol photons \(\text{m}^{-2} \text{s}^{-1}\) (Supplemental Fig. S3) or in high light at 1,000 \(\mu\)mol photons \(\text{m}^{-2} \text{s}^{-1}\) (Fig. 4) for 15 min. In both light conditions, the D1 protein (as confirmed by immunoblotting against the D1 protein) was primarily labeled by \[^{35}\text{S}]\text{Met/Cys}\) in wild-type plants (Fig. 4). These results indicate that impairment of both CEF and qE causes inhibition of the synthesis of the D1 protein under strong light conditions.
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DISCUSSION

Dual Role of CEF in Photoprotection of PSII

Impairment of CEF by the mutation of pgr5 caused acceleration of photoinhibition in both the presence and absence of chloramphenicol under strong light (Fig. 2B). However, impairment of qE by mutations of npq1 and npq4 accelerated photoinhibition only in the absence of chloramphenicol (Fig. 2B). These results indicate that impairment of CEF, but not qE, caused acceleration of the photodamage to PSII. Thus, CEF helps prevent photodamage per se to PSII in a qE-independent manner.

Inhibition of CEF caused a decrease in NPQ development under strong light (Fig. 2A). After transfer from strong light to darkness, the difference in the level of NPQ between the wild type and the pgr5 mutant was immediately abolished within 1 min (Fig. 2A). NPQ consists of qE, qI, and qT. Since qE but not qI and qT is quickly quenched after transfer to darkness (Baker, 2008), the decreased NPQ development in the pgr5 mutant might be attributable to suppressed qE. Impairment of qE by mutation of npq1 and npq4 accelerated photoinhibition, suggesting that acceleration of photoinhibition by impairment of CEF was at least partially attributable to suppression of qE. The qE mutants, npq1 and npq4, showed that impairment of qE accelerated photoinhibition without affecting the process of photodamage per se (Fig. 2B), indicating that the impairment of qE accelerates photoinhibition through inhibition of the repair of photodamaged PSII. The reduction of CEF by mutation of pgr5 and of qE by mutations of npq1 and npq4 suppressed the de novo synthesis of proteins, primarily the D1 protein, in thylakoid membranes in strong light (Fig. 4). This result suggests that CEF prevented inhibition of the repair of photodamaged PSII at the step of the de novo synthesis of thylakoid proteins in a qE-dependent manner.

CEF Reduces Direct Photodamage to PSII

The acceleration of photoinhibition by the impairment of CEF was at least partially attributable to an acceleration of the direct photodamage to PSII, as mentioned above. In pgr5 mutants, strong light has been demonstrated to damage PSI as well as PSII (Munekage et al., 2002). Therefore, it is possible that impairment of PSI could cause acceleration of photodamage to PSII through overreduction of the plastoquinone pool and PSII acceptor limitations. However, the fact that methyl viologen does not reduce the rate of photodamage in the pgr5 mutant (Supplemental Fig. S2B) indicates that acceleration of photodamage to PSII caused by a lack of CEF is not attributable to impairment of PSI. Both impairment of qE (Fig. 2B) and the production of hydrogen peroxide (Fig. 3B) had no effect on the process of photodamage per se to PSII (Fig. 3), indicating that the acceleration of photodamage upon the impairment of CEF is attributable to neither the impairment of qE nor the production of hydrogen peroxide. Furthermore, both interruption of the Calvin cycle that causes overreduction of photosynthetic electron transport (Hakala et al., 2005; Takahashi and Murata, 2005, 2006) and the exogenous supply of singlet oxygen (Nishiyama et al., 2004) have been demonstrated to have no effect on the process of photodamage. However, nigericin accelerated photoinhibition in both the presence and absence of chloramphenicol (Fig. 3B), indicating that impairment of the generation of ΔpH causes acceleration of photodamage to PSII. Similar results have been demonstrated with another uncoupler, NH4Cl, in the cyanobacterium Synechocystis (Drath et al., 2008). The acceleration of photodamage upon impairment of CEF, therefore, might be attributable to the disruption of the generation of a thylakoid ΔpH (Fig. 5).

The generation of a ΔpH decreases the pH of the thylakoid lumen. Since photodamage to PSII occurs primarily at the oxygen-evolving complex, which is on the luminal side of the thylakoid membrane (Hakala et al., 2005; Ohnishi et al., 2005), the change in the sensitivity of PSII to photodamage may be caused by the change in the pH of the thylakoid lumen. For
CEF generates a ΔpH that is important for qE development through activation of VDE and protonation of PsbS. Impairment of CEF suppressed qE development (Fig. 2A) and accelerated photoinhibition (Fig. 2B). These results suggest that the acceleration of photoinhibition upon impairment of CEF is at least partially attributable to suppression of qE development. Impairment of qE by the mutation of genes for VDE (npq1) and PsbS (npq4) accelerated photoinhibition under strong light (Fig. 2B). However, the effect of impairment of qE on photoinhibition was completely abolishes in the presence of chloramphenicol (Fig. 2B). These results indicate that the impairment of qE accelerates photoinhibition through inhibition of the protein synthesis-dependent repair of photodamaged PSII rather than through acceleration of photodamage to PSII. The impairment of both CEF and qE inhibited the de novo synthesis of the D1 protein, which is important for the repair of photodamaged PSII, under strong light (Fig. 4). These findings suggest that the acceleration of photoinhibition upon impairment of CEF is at least partially attributable to suppression of qE and that the development of qE is important for maintaining efficient protein synthesis (i.e. minimizing inhibitory effects) associated with the repair of PSII under strong light (Fig. 5).

qE involves thermal dissipation of energy from singlet excited state chlorophyll (1Chl*) in antenna complexes in PSII. This minimizes the production of triplet excited state chlorophyll molecules (3Chl*) that react with ground state oxygen (3O2) to form toxic singlet oxygen (1O2; Baroli and Niyogi, 2000; Triantaphylides et al., 2008). Furthermore, it has been suggested that qE down-regulates PSII activity and suppresses the production of hydrogen peroxide via superoxide through electron transfer to oxygen at PSI (Asada, 1999), although down-regulation of PSII was not observed in our study (Fig. 1B). In cyanobacteria, exogenous supply of singlet oxygen and hydrogen peroxide has been demonstrated to inhibit the repair of photodamaged PSII with no effect on the process of photodamage to PSII (Nishiyama et al., 2001, 2004). This inhibition of the repair occurs through inhibition of the de novo synthesis of PSII proteins, primarily the D1 protein, at the step of translation (Nishiyama et al., 2004, 2005; Kojima et al., 2007). Inhibition of the synthesis of the D1 protein upon impairment of qE and CEF, therefore, might be attributable to the production of reactive oxygen species (Fig. 5), particularly in strong light in which scavenging enzymes cannot detoxify reactive oxygen species at a sufficient rate. Indeed, CEF has been suggested to suppress the production of reactive oxygen species (Chow and Hope, 1998; Golding and Johnson, 2003).

When Do Plants Need pgr5-Dependent CEF to Avoid Photoinhibition?

The absorbed light energy for photosynthesis exceeds its rate of consumption in chloroplasts when plants are under environmental stresses such as low and high temperatures, drought, and high salinity (Takahashi and Murata, 2008). Under such conditions, the decreased amount of NADP+ causes the activation of pgr5-dependent CEF (Okegawa et al., 2008). CEF has also been demonstrated to be accelerated in stress conditions, such as strong light (Clarke and Johnson, 2001; Miyake et al., 2004; DalCorso et al., 2008), low CO2 (Munekage et al., 2002), and drought (Golding and Johnson, 2003; Golding et al., 2004), although not in drought when the air is enriched in CO2 and the redox posing for CEF is near optimal (Jia et al., 2008). Our results demonstrated that CEF generates an acidic lumen and a ΔpH and helps avoid photoinhibition by preventing photodamage to PSII in a qE-independent manner and minimizing inhibition of the repair of photodamaged PSII in a qE-dependent manner. Thus, CEF may be important in avoiding photoinhibition.
under conditions in which absorbed light is in excess of the requirements for photosynthesis.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis (Arabidopsis thaliana* ecotype Columbia) was used as the wild type in this study. We also used Arabidopsis CEF pathway (pgp7; Munekage et al., 2002) and NPQ (npq1 and npq4) mutants (Niyogi et al., 1998; Li et al., 2002). The wild type and all mutants were grown at 22°C in light at 100 μmol photons m⁻² s⁻¹ with a light/dark cycle of 10/14 h. At 3 to 4 weeks after germination, fully expanded leaves were used for experiments.

**Measurement of O₂ Production and CO₂ Uptake Rates**

A leaf disc (78.5 mm²) was punched from the leaf and immediately placed within the cuvette (1.5 mL). First the cuvette was filled with N₂ gas, then CO₂ and ^18O₂ gases were injected to create an atmosphere of approximately 3.5% CO₂ and 21% ^18O₂ in N₂. A leaf disc was exposed to light at 100, 500, or 1,000 μmol photons m⁻² s⁻¹ or kept in darkness at 25°C, and concentrations of ^18O₂ and CO₂ in the cuvette were monitored with an isotope ratio mass spectrometer (Micromass IsolPrime; Maxwell et al., 1998). The photosynthetic O₂ evolution rate was measured by the light-dependent production of ^18O₂. The photosynthetic CO₂ fixation rate was measured by the light-dependent consumption of CO₂.

**Measurement of Chlorophyll Fluorescence**

Chlorophyll fluorescence was measured with a pulse amplitude modulation fluorometer (PAM-2000; Heinz Walz). The Fm/F₀ was measured after incubation in darkness for 15 min. NPQ was calculated as (Fm - F₀)/Fm (Baker, 2008).

**Pulse Labeling of Proteins and Immunoblotting against the D1 Protein**

Leaf discs (78.5 mm²) were vacuum infiltrated with 1 mL of reaction medium (1 mM KH₂PO₄, pH 6.3, 0.1% [w/v] Tween 20, and 300 μCi of [³⁵S]Met/Cys [specific activity >1,000 Ci mmol⁻¹; BP Biomedical]) for 20 s. After vacuum infiltration, leaf discs were washed and floated on 1 mL of water. Leaf discs were exposed to light at either 100 or 1,000 μmol photons m⁻² s⁻¹ at 25°C for 15 min. The leaf discs were immediately frozen in liquid nitrogen, and thylakoid membranes were isolated (Aro et al., 1993a). Thylakoid proteins were solubilized in 100 μL of NuPAGE LDS sample buffer (Invitrogen) containing NuPAGE reducing agent (Invitrogen) by heating at 60°C for 5 min. The solution was then centrifuged at 2,500g for 5 min, and the supernatant (10 μL) was electrophoretically separated on a 4%–12% Bis-Tris gel; Invitrogen) with NuPAGE MES-DS running buffer for approximately 48 h. The gels were scanned on a PhosphorImager (Molecular Dynamics). The protein bands on PVDF membrane were detected by Coomasie Brilliant Blue (GelCode Blue stain reagent; Pierce). After Coomasie Brilliant Blue was removed from PVDF by incubation in 95% methanol, the D1 protein on PVDF was immunologically detected with an antibody specific to the D1 protein (Agrisera) and an alkaline phosphatase conjugate substrate kit (Bio-Rad).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Effect of antimycin A on NPQ and photoinhibition.

**Supplemental Figure S2.** Effect of methyl viologen and nigericin on NPQ and photoinhibition in the pgp5 mutants.

**Supplemental Figure S3.** Effect of impairments of CEF and NPQ on the de novo synthesis of PSII proteins in low light.

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