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Reduction-Induced Suppression of Electron Flow (RISE) Is Relieved by Non-ATP-Consuming Electron Flow in Synechococcus elongatus PCC 7942

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Photosynthetic organisms oxidize P700 to suppress the production of reactive oxygen species (ROS) in photosystem I (PSI) in response to the lower efficiency of photosynthesis under high light and low CO2 conditions. Previously, we found a positive relationship between reduction of plastoquinone (PQ) pool and oxidation of P700, which we named reduction-induced suppression of electron flow (RISE). In the RISE model, we proposed that the highly reduced state of the PQ pool suppresses Q-cycle turnover to oxidize P700 in PSI. Here, we tested whether RISE was relieved by the oxidation of the PQ pool, but not by the dissipation of the proton gradient (ΔpH) across the thylakoid membrane. Formation of ΔpH can also suppress electron flow to P700, because acidification on the luminal side of the thylakoid membrane lowers oxidation of reduced PQ in the cytochrome b6/f complex. We drove photosynthetic electron transport using H2O2-scavenging peroxidase reactions. Peroxidase reduces H2O2 with electron donors regenerated along the photosynthetic electron transport system, thereby promoting the formation of ΔpH. Addition of H2O2 to the cyanobacterium Synechococcus elongatus PCC 7942 under low CO2 conditions induced photochemical quenching of chlorophyll fluorescence, enhanced NADPH fluorescence and reduced P700. Thus, peroxidase reactions relieved the RISE mechanism, indicating that P700 oxidation can be induced only by the reduction of PQ to suppress the production of ROS in PSI. Overall, our data suggest that RISE regulates the redox state of P700 in PSI in cooperation with ΔpH regulation.

Keywords: P700 oxidation, photosynthesis, reactive oxygen species, plastoquinone, Q-cycle

INTRODUCTION

Oxygenic phototrophs adjust photon energy utilization to environmental conditions in an attempt to alleviate photo-oxidative damage. Solar photon energy often exceeds photosynthetic CO2 assimilation needs, which has the potential to overflow into O2 in photosystem I (PSI), thereby generating reactive oxygen species (ROS), including superoxide anion radical, hydroxyl radical,
and singlet oxygen (Satoh, 1970; Sonogke, 1996; Cazzaniga et al., 2012; Sejima et al., 2014; Takagi et al., 2016b). Because of their high reactivity, ROS immediately obliterate PSI photochemical activity and the inactivated PSI takes days to weeks to recover (Kudoh and Sonogke, 2002; Zivcak et al., 2015). The photo-oxidative damage in PSI, derived from ROS high reactivity, can be easily induced by repetitive short-pulse illumination, which instantaneously fills the photosynthetic electron transport system with electrons (Sejima et al., 2014). The inactivation of PSI is suppressed if the reaction center chlorophyll (Chl) in PSI, P700, is kept oxidized (Sejima et al., 2014; Shimakawa et al., 2016b, 2017a; Takagi et al., 2017b). Photosynthetic organisms flexibly oxidize P700 in response to high light intensity and low CO2 conditions, in an attempt to suppress ROS production (Badger and Schreiber, 1993; Golding and Johnson, 2003; Miyake et al., 2005; Sejima et al., 2014; Shimakawa et al., 2016b, 2017a; Takagi et al., 2017b). The oxidation of P700 strictly indicates that the re-reduction of oxidized P700 by electrons from PSI is prevented, but here we use the simple term “P700 oxidation” for this physiological response. P700 oxidation is a universal strategy used by photosynthetic organisms to decrease the risk of ROS production by lowering the amount of ground state P700, the source of excess electrons and energy. That is why photo-oxidative damage in PSI rarely occurs.

Oxidation of P700 in PSI is regulated by a variety of molecular mechanisms (P700 oxidation system). These are categorized as either acceptor-side mechanisms, i.e., those which safely dissipate excess electrons and energy through electron transport in order to relax the limitation of the electron acceptor side of PSI (alternative electron transport), or donor-side mechanisms, i.e., those which suppress electron transport into PSI (Shimakawa et al., 2016b, 2017a; Takagi et al., 2017b). In the case of acceptor-side mechanisms, photorespiration prepares a major alternative electron sink in land plants, except for C4 plants (Takagi et al., 2016a; Hanawa et al., 2017). Furthermore, flavodiiron protein mediates alternative electron transport to oxidize P700 in cyanobacteria (Helman et al., 2003; Allahverdiyeva et al., 2013; Shimakawa et al., 2015, 2016b), chlorophytes (Chaux et al., 2017), bryophytes (Gerotto et al., 2016; Shimakawa et al., 2017a), and probably pteridophytes and gymnosperms (Zhang et al., 2009; Takagi et al., 2017b). Both P700 oxidation systems on the electron acceptor side require O2 as the electron acceptor (Helman et al., 2003; Hayashi et al., 2014; Sejima et al., 2016; Hanawa et al., 2017). On the donor side, P700 oxidation is known to have a strong relationship with the proton gradient (ΔpH) across the thylakoid membrane. Studies on isolated chloroplasts have shown that acidification on the luminal side of the thylakoid membrane suppresses electron transport in the cytochrome b6/f complex (Cyt b6/f) (Tikhonov et al., 1981; Nishio and Whitmarsh, 1993). This has been subsequently supported by in vivo physiological measurements on intact plant leaves (Takizawa et al., 2008; Rott et al., 2011; Takagi et al., 2017a) and living cyanobacterial cells (Trubitsin et al., 2003). Additionally, energy-dependent non-photochemical quenching (qE or qZ) is activated by ΔpH to dissipate excess photon energy as heat at photosystem II (PSII) in plants, algae, and cyanobacteria (Niyogi and Truong, 2013; Stamatakis and Papageorgiou, 2014; Ruban, 2016). Furthermore, H2O oxidation in PSII is inhibited at low pH on the luminal side of the thylakoid membrane (Krieger et al., 1993). These mechanisms help alleviate the pressure of electron transport on the donor side of PSI and contribute to P700 oxidation.

Recently, Shaku et al. (2016) identified a novel P700 oxidation mechanism operating on the donor side of PSI: reduction-induced suppression of electron flow (RISE). In photosynthetic linear electron flow (LEF) on the thylakoid membrane, plastoquinol (PQH2) is oxidized to plastoquinone (PQ) in Cyt b6/f, where the Q-cycle operates (Figure 1; Kallas, 1994; Tikhonov, 2014). In the Q-cycle, PQH2 donates one electron to an iron-sulfur cluster at the PQH2 oxidation site (Qp site) in Cyt b6/f; cytochrome f (Cyt f) accepts the electron from the iron-sulfur cluster. The electron in the PQ semiquinone remaining at the Qp site is transferred to a PQ at the PQ reduction site (Qh site) in Cyt b6/f. The PQ in the one-electron-reduced form at the Qh site accepts the second electron from PSI and becomes reduced to PQH2 at the Qp site in Cyt b6/f. When two molecules of PQH2 are oxidized at the Qh site in Cyt b6/f, two electrons are transported to Cyt f sequentially and the other two circulate within Cyt b6/f to produce one molecule of PQH2 at the Qh site (Figure 1; Kallas, 1994; Tikhonov, 2014). Theoretically, unless PQ is supplied to the Qh site, the Q-cycle cannot operate and the reduction of Cyt f is suppressed. Shaku et al. (2016) showed that a reduction of the PQ pool in the Synechococcus elongatus PCC 7942 (S. elongatus) flavodiiron protein-deficient mutant causes suppression of electron transport from PQH2 to PSI, which in turn results in the accumulation of oxidized P700. That is, in the mutant, the Q-cycle function is suppressed due to the shortage of PQ supplied for the Qh site in Cyt b6/f, resulting in suppressed LEF under CO2 limitation. Therefore, the mutant can survive in an air-equilibrated condition (Shimakawa et al., 2016b).

As described above, on the electron donor side of PSI, two molecular mechanisms for P700 oxidation can function: the ΔpH-dependent suppression of PQH2 oxidation in Cyt b6/f and the suppression of the Q-cycle, which depends on the accumulation of PQH2 (RISE). In order to demonstrate that RISE is regulated by electron-sink activity, in the present study, we tested whether RISE is relieved by a non-ATP-consuming metabolic pathway. A previous report by Shaku et al. (2016) showed that P700 oxidation induced by RISE is suppressed by addition of NaHCO3 to the cyanobacterial cells. Simultaneously, NaHCO3 starts CO2-dependent O2 evolution, i.e., photosynthesis. Carbon assimilation consumes ATP in addition to NADPH and dissipates ΔpH formed across the thylakoid membrane. Based on these facts, we could not exclude the possibility that the suppression of P700 oxidation might be driven by the increased activity of Cyt b6/f under conditions of dissipated ΔpH. Thus, we tried to show that a non-ATP-consuming metabolic pathway, electron sink, prevents RISE from operating. We investigated the effect of H2O2-dependent electron flow as a non-ATP-consuming metabolic pathway on the relaxation of RISE in S. elongatus.

Cyanobacteria detoxify H2O2 using catalase and peroxidase reactions (Miyake et al., 1991). The peroxidase reaction uses electron donors such as NADPH (Miyake et al., 1991;
Shimakawa et al. Reduction-Induced Suppression of Electron Flow

FIGURE 1 | Proposed model of reduction-induced suppression of electron flow (RISE). (A) Q-cycle in high electron-sink conditions (i.e., α and γ in Figure 2). The activity of photosynthetic linear electron flow (LEF) is high. Plastoquinone (PQ) is reduced to plastoquinol (PQH$_2$) with electrons from photosystem II (PSII) and the Q$_n$ site in the cytochrome $b_6$/$f$ complex (Cyt $b_6$/$f$). Then, PQH$_2$ is oxidized at the Q$_p$ site in Cyt $b_6$/$f$. (B) The Q-cycle in low electron-sink conditions (i.e., β in Figure 2). The activity of LEF is low, and PQ pool is highly reduced, leading the suppression of the Q cycle in Cyt $b_6$/$f$ to oxidize P700 in photosystem I (PSI). Blue, brown, and yellow ellipsoid respectively indicate plastocyanin (or cytochrome c$_6$), ferredoxin, and ferredoxin-NADP$^+$ reductase. Red and blue lines indicate the transports of electrons and protons, and dashed red line shows the suppressed electron flow.

Yamamoto et al., 1999). For continuous scavenging of H$_2$O$_2$, these electron donors are regenerated by LEF (Miyake et al., 1991). Therefore, addition of H$_2$O$_2$ to cyanobacterial cells induces both photochemical quenching of Chl fluorescence and O$_2$ evolution in the light (Miyake et al., 1991). That is, H$_2$O$_2$-dependent peroxidase reaction drives LEF, which is also observed in isolated intact chloroplasts from plant leaves (Schreiber and Neubauer, 1990; Miyake and Asada, 1992). This H$_2$O$_2$-dependent peroxidase reaction does not consume ATP. Therefore, if the H$_2$O$_2$-dependent peroxidase reaction results in RISE shutting off, then it would indicate that electron sink activity regulates RISE.

The NADPH redox level provides the information of the dynamic property of the electron acceptor side of PSI, which can be evaluated as blue green fluorescence using a Dual-PAM-100 instrument (Heinz Walz, Effeltrich, Germany; Mi et al., 2000; Schreiber and Klughammer, 2009; Kauny and Sétif, 2014; Holland et al., 2015; Shaku et al., 2016). Recently, Holland et al. (2015) investigated the dynamic response of the NADPH redox level to CO$_2$ limitation in the cyanobacterium *Synechocystis* sp. PCC 6803. Limiting CO$_2$ causes the suppression of the Calvin-Benson cycle to lower the efficiency of the consumption of NADPH, resulting in the reduction of the NADP$^+$ pool (Holland et al., 2015). However, the NADP$^+$ pool is not fully reduced even under CO$_2$ limitation, indicating that not only the consumption but also the production of NADPH is suppressed in response to CO$_2$ limitation. Overall, the abovementioned molecular mechanisms for P700 oxidation contribute to keep part of the NADP$^+$ pool oxidized, and it is expected that the pool will...
be more reduced when the suppression of electron transport in Cyt \( b_6/f \) is relaxed.

MATERIALS AND METHODS

Growth Conditions and Chl a Determination

Cyanobacterial cultures were maintained on BG-11 solid medium (Allen, 1968) under continuous fluorescent lighting (25° C, 50 µmol photons m\(^{-2}\) s\(^{-1}\)). For all physiological experiments, cells from the cultures were inoculated into BG-11 liquid medium (initial OD\(_{750}\) 0.1–0.2) and grown on a rotary shaker (100 rpm) under a light/dark cycle (light period: 16 h, at 25° C, 150 µmol photons m\(^{-2}\) s\(^{-1}\); dark period: 8 h, at 23° C), at 2% (v/v) [CO\(_2\)]. Optical density of the medium at 750 nm was measured with a spectrophotometer (U-2800A, Hitachi, Tokyo, Japan). Cells from the early exponential growth phase (OD\(_{750}\) 2–3) were used for the experiments.

For Chl measurements, cells from 0.1 to 1.0 mL cultures were harvested by centrifugation and resuspended by vortexing in 1 mL 100% (v/v) methanol. After incubation at room temperature for 5 min, the suspension was centrifuged at 10,000 × g for 5 min. Total Chl \( a \) was spectrophotometrically determined from the supernatant (Grimme and Boardman, 1972).

Measurement of Chl and NADPH Fluorescence, and P700 Absorbance

Both Chl and NADPH fluorescence were simultaneously measured with a Dual-PAM-100 instrument (Heinz Walz, Effeltrich, Germany) at room temperature (25° C ± 2° C). The reaction mixtures (2 mL) contained 50 mM HEPES (pH 7.5) and the cells (10 µg Chl mL\(^{-1}\)). During the measurement, the reaction mixture was stirred with a magnetic micro stirrer. Photon flux density of red actinic light (AL, LED with peak emission at 635 nm) was 200 µmol photons m\(^{-2}\) s\(^{-1}\). The values of incident quantum yield of PSII, \( Y(II) \), which reflect the apparent electron flux in LEF (Genty et al., 1989; Shimakawa et al., 2017b), were calculated from Chl fluorescence parameters, \( \Phi_n \), the signal level for fully oxidized NADP\(^+\) pool; \( N_n \), the signal level for fully reduced NADP\(^+\) pool; \( N_{m} \), the current signal for the relative extent of NADP\(^+\) reduction.

Measurement of P700 absorbance was performed with a Dual-PAM-100 instrument (Heinz Walz, Effeltrich, Germany) in almost the same conditions as described for Chl and NADPH fluorescence analysis. The redox state of P700 was determined according to the method of Klughammer and Schreiber (2008). In this procedure, \( P_m \) = maximum P700 photo-oxidation level, obtained by a saturated pulse light under far-red illumination; \( P = \) oxidation level of P700 under AL; \( P_m' \) = maximum oxidation level of P700, obtained by a saturation pulse under AL illumination; \( \gamma(1) = (P_m - P)/P_m = \) incident quantum yield of photochemical energy conversion; \( \gamma(ND) = P/P_m = \) quantum yield of non-photochemical energy dissipation due to a donor-side limitation and \( \gamma(NA) = (P_m - P)/P_m = \) quantum yield of non-photochemical energy dissipation due to an acceptor-side limitation. The sum of the three factors \( [\gamma(1) + \gamma(NA) + \gamma(ND)] = 1 \). For the determination of these parameters, a 300 ms saturation pulse (10,000 µmol photons m\(^{-2}\) s\(^{-1}\)) was used, and the stirrer was turned off 5 s before the saturation pulse was applied.

Measurement of \( O_2 \) Exchange

Uptake and evolution of \( O_2 \) were measured with a Clark-type \( O_2 \) electrode at 25° C (Hansatech, King’s Lynn, United Kingdom) with a high time resolution (Sejima et al., 2016; Hanawa et al., 2017). The \( O_2 \) amount in the reaction mixture were obtained in an analog recorder with the signal amplitude and the time scale properly adjusted as in Shimakawa et al. (2016a). The reaction mixture (2 mL) contained 50 mM HEPES (pH 7.5) and the cyanobacterial cells (10 µg Chl mL\(^{-1}\)). Red AL (620 < λ < 695 nm, 200 µmol photons m\(^{-2}\) s\(^{-1}\)) was provided by a halogen lamp (Xenophot HLX 64625, Osram, München, Germany) with an LS2 light source (Hansatech, King’s Lynn, United Kingdom). During the measurement, the reaction mixture was stirred with a magnetic micro stirrer.

Generation of Mutants

The \textit{S. elongatus katG} deficient mutant (Synpc7942_1656) was generated by the method of Shaku et al. (2016). To obtain the knock-out construct (Supplementary Figure S1A), polymerase chain reaction (PCR) was used to amplify the genomic region encoding \( katG \) with a primer set (f, TTCAATTTTGGCTGGCGTCA; r, GCATT CATCACCCTTGCTCCA). The PCR product was then cloned into the pGEM-T Easy vector (Promega, Tokyo, Japan). The recombinant plasmid was linearized and amplified by inverse PCR with a primer set (f, TTGGCCTTGGAATATGGCAGTGGAACCGAT; r, AAACCGCACCAGTACAGAAGCTGGCAGCAATAC), and then applied to the In-Fusion reaction (Takara, Shiga, Japan) with a kanamycin-resistance gene (\( Kan^R \)) derived from pUC4K vector.
Transformation of wild type *S. elongatus* was performed by the standard procedure (Williams, 1988), and the mutant, ΔkatG, was selected on BG-11 agar plates containing kanamycin (20 μg mL⁻¹). Complete segregation was confirmed by PCR (Supplementary Figure S1B).

### RESULTS AND DISCUSSION

#### Experimental Scheme

In general, CO₂ consumption under constant light suppressed cyanobacterial photosynthesis, as observed in the decrease in incident quantum yield of PSII, Y(II), which is estimated from Chl fluorescence analysis (Figure 2A; Hayashi et al., 2014; Shimakawa et al., 2015, 2016b). Addition of NaHCO₃ to the cyanobacterial cells restored photosynthesis, as observed in the increase in Y(II) (Figure 2A; Hayashi et al., 2014; Shimakawa et al., 2015, 2016b). Experimentally, this can be observed as a three-phase (α, β, and γ; Figure 2A) time course. Reduction state of the PQ pool, reflected in F₆/F₇, responds to these three phases. In phase α, which is low photosynthetic rate is observed, PQ is oxidized, and in phase β, which is low photosynthetic rate is observed, PQ is reduced, as inferred from the increase in the Chl fluorescence parameter F₆/F₇ (Hayashi et al., 2014). The reduced state of PQ is relieved in phase γ. Furthermore, the oxidation state of P700, reflected in quantum yield of non-photochemical energy dissipation due to donor-side limitation, Y(ND), from P700 absorbance analysis, also responds to these three phases in a similar fashion to the redox state of PQ (Figure 2C; Shaku et al., 2016; Shimakawa et al., 2016b). We refer to these responses of Y(II), F₆/F₇, and Y(ND) as RISE (Shaku et al., 2016).

We can explain RISE using the model of the Q-cycle, as shown in Figure 1. In phase α and γ, the Q-cycle operates in a high-electron sink condition (Figure 1A). The occupancy of the oxidized form of PQ is high, and an electron from the reduced form of PQ at the Q₀ site in Cyt b₅/f can be rapidly transferred into the Qₐ site for the reduction of PQ; that is, the high-electron sink condition makes Q-cycle turnover rapid (Figure 1A). Conversely, in phase β, which is low electron sink activity is low (i.e., low-electron sink condition; Figure 1B). Q-cycle turnover is slowed down. A low-electron sink condition reduces PQ, as inferred from the increase in F₆/F₇ (Figure 2B; Hayashi et al., 2014; Shaku et al., 2016); that is, the ratio of PQ to PQH₂ decreases and the efficiency of the donation of electrons from Qₐ to Q₀ sites decreases. This results in the suppression of Q-cycle turnover, which in turn suppresses the reduction of cytochrome f, plastocyanin (or cytochrome c₅), and eventually oxidizes P700 (Figure 1B). This is the mechanism for oxidation of P700 in phase β (Figure 2C). We refer to this modulation of Q-cycle turnover for P700 oxidation as RISE (Shaku et al., 2016).

In the present study, we aimed to further characterize the possible mechanism underlying RISE. In our previous report (Shaku et al., 2016), we activated photosynthesis with NaHCO₃ to relax RISE. The activation of photosynthesis dissipates ΔpH across the thylakoid membrane by the consumption of ATP. The acidification of the lumen also suppresses the oxidation activity of PQH₂ in Cyt b₅/f (Tikhonov et al., 1981; Nishio and Whitmarsh, 1993), similar to RISE. We tried to relieve RISE by stimulating electron flow in phase β to prove that RISE is regulated by the redox state of PQ and the electron sink activity. We used H₂O₂-dependent electron flow (Miyake et al., 1991; Miyake and Asada, 1992). Cyanobacteria have several peroxidases, which utilize electron donors to reduce H₂O₂ to H₂O (Miyake et al., 1991; Yamamoto et al., 1999). For continuous scavenging of H₂O₂, the oxidized electron donor is reduced by the photosynthetic electron transport system (Miyake and Asada, 1992). That is, addition of H₂O₂ to cyanobacterial cells drives LIF. The H₂O₂-dependent electron flow induces ΔpH across the thylakoid membrane because no ATP is consumed (Schreiber and Neubauer, 1990; Miyake and Asada, 1992).

To elucidate the occurrence of RISE and the response to the electron sink activity in *S. elongatus*, we simultaneously evaluated Chl and NADPH fluorescence after the establishment of phase β by the consumption of CO₂ in the reaction mixture. In phase β, F₆/F₇ is kept at higher values (Figure 2B). Addition of an electron acceptor to the photosynthetic electron transport system should decrease F₆/F₇. The decrease in F₆/F₇ would show the acceleration of electron flow driven by the electron acceptor.

#### Relaxing of RISE and Acceleration of Linear Electron Flow by Exogenous NaHCO₃ in *S. elongatus*

Cells of *S. elongatus* were illuminated with red AL (20 µmol photons m⁻² s⁻¹) without the supplement of an inorganic carbon source. Steady-state Chl fluorescence yield (i.e., F₇) immediately increased in response to AL and then gradually decreased to a constant value during phase α (Figure 3A). Thereafter, F₆ dramatically increased (Figure 3A), accompanying the decrease in Y(II) from 0.32 ± 0.04 at 20 min in phase α to 0.023 ± 0.003 at 40 min in phase β (mean ± standard deviation, n = 3). In this study, we sought to evaluate the NADPH redox level during the measurements following the method by Schreiber and Klughammer (2009). Because the base line signal of the NADPH fluorescence can drift during a long-term measurement (Schreiber and Klughammer, 2009; Kauny and Sétif, 2014; Holland et al., 2015), the maximum reduction level of NADP⁺ pool, defined as Nₓ, was periodically determined by applying a saturated short-pulse light (1 s, 10,000 µmol photons m⁻² s⁻¹; Figure 3B). Additionally, the maximum oxidation level of NADP⁺ pool, defined as N₀, was determined in the dark just after applying the short-pulse light (Figure 3B). The current NADPH fluorescence signal (Nₜ) was continuously monitored. That is, the oxidation fraction of NADP⁺ pool was estimated as (Nₜ−N₀)/(Nₓ−N₀) during the measurements (Schreiber and Klughammer, 2009). During the transition to CO₂ limitation (from phases α to β), we periodically determined (Nₓ−Nₜ)/(Nₓ−N₀) and found that the redox level of NADP⁺ pool did not change in response to CO₂ limitation (Figure 3C). On the other hand, adding the Calvin-Benson cycle inhibitor glycolaldehyde caused the decrease in (Nₓ−Nₜ)/(Nₓ−N₀) (Figure 3D). These facts indicate that
NADP⁺ pool is not fully reduced even under CO₂ limitation, which is consistent with the preceding report (Holland et al., 2015). In response to CO₂ limitation, LEF is suppressed in Cyt b₆/f and P700 is kept oxidized (Shaku et al., 2016; Shimakawa et al., 2016b), which should lower the production of NADPH to save the oxidation fraction of NADP⁺ pool. On the other hand, it is expected that the more severe suppression of the Calvin-Benson cycle can cause the reduction of NADP⁺ pool, which is supported by the effect of glycolaldehyde on the NADPH fluorescence signal (Figure 3D; Holland et al., 2015). That is, the NADPH redox level severely depends on the degree of the suppression of the Calvin-Benson cycle, which might cause a different response of the NADPH redox level to CO₂ limitation (Holland et al., 2015). In this study, we note that electron flow to the oxidized P700 in PSI was suppressed strongly enough not to reduce NADP⁺ in phase β. We refer to the suppressed electron flow to P700 in phase β as RISE.

We evaluated the relaxation of RISE by adding NaHCO₃ to the cells of S. elongatus in phase β. NaHCO₃-dependent relief of RISE would be expected to increase the electron flow to NADP⁺ by oxidizing PQH₂ and/or dissipating ΔpH for ATP synthesis. We added 50 µM NaHCO₃ to S. elongatus in phase β and observed photochemical quenching reflected as a rapid decrease in F₅ (Figure 4). That is, PQH₂ was oxidized. We determined Y(II) at three points in time during the experiment (Figure 4): I, before NaHCO₃ was added; II, while Chl fluorescence was photochemically quenched; and III, after F₅ returned to a high level (0.028 ± 0.006, 0.12 ± 0.02, and 0.029 ± 0.007, respectively [mean ± standard deviation, n = 3]). The results showed that NaHCO₃ enhanced electron flux in LEF, which led us to expect that stimulated photosynthetic CO₂ assimilation would enhance NADPH consumption (Hayashi et al., 2014). Additionally, NADPH fluorescence rapidly increased by the addition of NaHCO₃ and then gradually decreased (Figure 4). These results suggest that addition of NaHCO₃ transiently reduced NADP⁺ and then gradually oxidized NADPH. As shown by the pattern of Chl fluorescence, PQH₂ accumulated in phase β was oxidized to relieve RISE, which accelerated the electron flow to NADP⁺. Oxidation efficiency of NADPH in NaHCO₃-stimulated photosynthesis was overwhelmed by the reduction efficiency of NADP⁺ in LEF, accelerated by the relaxing of RISE. This would explain why the oxidation of NADPH was not observed upon NaHCO₃ addition to the cells. Overall, the addition of NaHCO₃ relaxed RISE. However, we could not conclude whether the oxidation of PQH₂ or the dissipation of ΔpH across the thylakoid membrane relaxed RISE.

**FIGURE 2** | Time courses of the incident quantum yield of PSII, Y(II) (A), the chlorophyll fluorescence parameter inferring the reduction of the plastoquinone (PQ) pool, F₅/F₆ (B), and the electron donor-side limitation of photosystem I, Y(ND) (C) in response to CO₂ limitation in Synechococcus elongatus PCC 7942. Cyanobacterial photosynthesis starts at 0 min with an actinic light. Thereafter, Y(II) decreases due to a shortage of CO₂, which is defined as the transition from phase α to β. The addition of CO₂ in the form of NaHCO₃ restores photosynthetic activity (phase γ). Data are mean ± the standard deviation of three measurements (technical replicates).

## Relaxing of RISE and Acceleration of Linear Electron Flow by Exogenous H₂O₂ in S. elongatus

Next, we studied the effect of exogenous H₂O₂ on Chl and NADPH fluorescence in phase β in S. elongatus. The measurement was performed in the presence of hydroxylamine (25 µM), a catalase inhibitor. Upon addition of 50 µM H₂O₂, F₅ decreased, which resulted in Y(II) values of 0.027 ± 0.004, 0.120 ± 0.011, and 0.027 ± 0.007 at points in time I, II, and III, respectively (mean ± standard deviation, n = 3) (Figure 5). In other words, photochemical quenching occurred in response to the addition of H₂O₂ in phase β. Thereafter, F₅ increased with the consumption of H₂O₂ (Figure 5; Miyake et al., 1991). NADPH...
fluorescence immediately decreased in response to the addition of H$_2$O$_2$, and then gradually increased (Figure 5). The increase in NADPH fluorescence was accompanied by enhanced electron flux through LEF, as observed in the increase in Y(II). Thereafter, NADPH fluorescence decreased with the consumption of H$_2$O$_2$, as evidenced by the increase in $F_o$ (Figure 5).

Simultaneously, we analyzed the response of the redox state of P700 in PSI to the addition of H$_2$O$_2$ to the cyanobacterial cells.
in phase β. Compared with the redox state of P700 at point I, the addition of H\textsubscript{2}O\textsubscript{2} increased incident quantum yield of PSI, Y(I), and decreased Y(ND), at point II (Table 1). Thereafter, both Y(I) and Y(ND) recovered to the original values at point III (Table 1). Quantum yield of non-photochemical energy dissipation due to acceptor-side limitation Y(NA), did not show any changes (Table 1). These results indicate that in the scavenging of H\textsubscript{2}O\textsubscript{2} the electron flux to NADP\textsuperscript{+} was enhanced, although P700\textsuperscript{+} was reduced, that is, the scavenging of H\textsubscript{2}O\textsubscript{2} induced the oxidation of PQH\textsubscript{2}, which accompanied enhanced electron flux to NADP\textsuperscript{+} transiently. This shows that RISE was indeed relaxed only by the oxidation of PQH\textsubscript{2}, as observed in the decrease in Y(ND).

In cyanobacteria, H\textsubscript{2}O\textsubscript{2} can be scavenged via two types of reactions, (i) catalase and (ii) peroxidase (Miyake et al., 1991; Tichy and Vermaas, 1999; Yamamoto et al., 1999; Stork et al., 2009):

\[ H_2O_2 \rightarrow 1/2O_2 + H_2O \]  
(1)

\[ H_2O_2 + Red \cdot H_2 \rightarrow 2H_2O + Oxi \]  
(2)

Catalase detoxifies H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O and O\textsubscript{2} is evolved, whereas peroxidases utilize electron donors (indicated by Red and Oxi) as the reduced and oxidized forms of electron donors. For example, in S. elongatus, thioredoxin functions as the electron donor in both thioredoxin peroxidase and peroxiredoxin Q reactions (Stork et al., 2009). Thioredoxin is reduced by NADPH-thioredoxin reductase with NADPH (iii) (Miyake et al., 1991; Yamamoto et al., 1999). The NADP\textsuperscript{+} produced in the peroxidase reactions is reduced back to NADPH in the photosynthetic electron transport system (iv); the scavenging of H\textsubscript{2}O\textsubscript{2} by the peroxidase reactions is coupled with LEE, which is linked to the evolution of O\textsubscript{2} in PSI (Miyake et al., 1991; Yamamoto et al., 1999; Miyake and Asada, 2003).

\[ Oxi + NADPH + H^+ \rightarrow Red \cdot H_2 + NADP^+ \]  
(3)

\[ H_2O + NADP^+ \rightarrow 1/2O_2 + NADPH + H^+ \]  
(4)

\[ H_2O_2 \rightarrow 1/2O_2 + H_2O \]  
(5)

Thus, exogenous H\textsubscript{2}O\textsubscript{2} functions as the alternative electron acceptor to stimulate LEE, as supported by chemical quenching of Chl fluorescence (Figure 5). The rapid decrease in NADPH fluorescence immediately after H\textsubscript{2}O\textsubscript{2} addition might be due to consumption of NADPH via the abovementioned peroxidase reactions, with the accumulation of the oxidized form of the electron donors, NADPH fluorescence increased by the relaxation of RISE.

We conclude that some parts of electron transport suppression in phase β in S. elongatus, depend only on the redox state of the PQ pool, but not on ΔpH (Figure 5). Exogenous H\textsubscript{2}O\textsubscript{2} accelerated LEE to reduce NADP\textsuperscript{+}; the gradual increase in NADPH fluorescence was clearly related to the chemical quenching of Chl fluorescence (Figure 5). From the abovementioned formulae, peroxidase-dependent H\textsubscript{2}O\textsubscript{2}-scavenging

| \( Y(I) \) | \( Y(ND) \) | \( Y(NA) \) |
|-----------|-----------|-----------|
| I         | 0.36 ± 0.03 | 0.55 ± 0.01 | 0.09 ± 0.02 |
| II        | 0.51 ± 0.03 | 0.37 ± 0.05 | 0.12 ± 0.02 |
| III       | 0.34 ± 0.01 | 0.56 ± 0.02 | 0.10 ± 0.03 |
reactions do not require ATP; ΔpH formation is rather promoted (Schreiber and Neubauer, 1990). In other words, the oxidized electron donor produced in the peroxidase reactions effectively relieved RISE.

The acceleration of LEF by exogenous H$_2$O$_2$ was evaluated also by measuring O$_2$ evolution rate in _S. elongatus_. Scavenging of H$_2$O$_2$ by the peroxidase reactions caused O$_2$ evolution at PSII, because the regeneration of the reductants is coupled to LEF (v). H$_2$O$_2$-dependent O$_2$ evolution was measured in the presence of hydroxylamine (25 μM), as the activity of catalase in _S. elongatus_ is so large that it masks O$_2$ evolution derived from the peroxidase reactions (Shimakawa et al., 2017b). Unfortunately, we could not completely inhibit the catalase activity of _S. elongatus_ wild type by hydroxylamine at 25 μM. A portion of the catalase-dependent O$_2$ evolution rate was detected in the dark even in the presence of hydroxylamine (15 ± 5 μmol O$_2$ mg$^{-1}$ Chl h$^{-1}$, mean ± standard deviation, n = 3), or approximately 5% of the intact activity (Figure 6). To solve this problem, we constructed an _S. elongatus_ mutant (ΔkatG) deficient in the dominant gene encoding catalase (Supplementary Figure S1). In the dark, the O$_2$ evolution by the catalase reaction was not observed in ΔkatG in the presence of hydroxylamine at 25 μM. In the dark, the addition of H$_2$O$_2$ to the wild type of cyanobacterial cells rapidly induced the evolution of O$_2$, indicating instantaneous decomposition of H$_2$O$_2$ to H$_2$O and O$_2$ by catalase (Figure 6); H$_2$O$_2$ rapidly entered the cells. However, induced O$_2$ evolution proceeded more slowly in the presence of hydroxylamine in both, wild type and ΔkatG, compared with the catalase reaction (Figure 6). The retardation of O$_2$ evolution induction reflects the slow relaxation of RISE, which is consistent with the slow decrease in F$_3$ and the slow increase in NADPH fluorescence (Figure 5), probably due to the low production rate of the oxidized form of electron donors for the peroxidase reaction. We evaluated the relationship of Y(II) to overall O$_2$ evolution (the sum of O$_2$ evolution rate and dark respiration rate [Rd]) in wild type and ΔkatG of _S. elongatus_, validating the relationship between scavenging of H$_2$O$_2$ and LEF (Figure 7). Linearity of the relationship was recognized in ΔkatG, which supported the idea that H$_2$O$_2$-dependent O$_2$ evolution rate reflected peroxidase reaction scavenging H$_2$O$_2$ coupled to photosynthetic electron transport reactions.

We need to point out that the acceleration of LEF by H$_2$O$_2$ via the peroxidase reaction might not occur as long as the P700 oxidation system and the catalase-dependent H$_2$O$_2$-scavenging reaction are in operation. Firstly, the production of H$_2$O$_2$ in PSI would be strictly suppressed where P700 is oxidized. Especially flavodiiron protein dissipates excess photon energy in PSI to prevent the production of superoxide anion radical, which significantly decreases the physiological relevance of H$_2$O$_2$-dependent LEF in cyanobacteria (Helman et al., 2003; Allahverdiyeva et al., 2013; Weenink et al., 2015; Shimakawa et al., 2016b). Secondly, cyanobacteria show the greater scavenging activity of H$_2$O$_2$ in the catalase reaction, compared with the peroxidase reaction (Figure 6) (Shimakawa et al., 2017b). Therefore, we eliminated the effects of catalase on the cells with hydroxylamine and the mutant ΔkatG to create the situation where peroxidase-dependent H$_2$O$_2$-scavenging reactions operate in _S. elongatus_. Overall, in this study, we used the peroxidase reaction in _S. elongatus_ as an experimental tool for verification of RISE in phase β in _S. elongatus_, because the peroxidase reaction functions with photosynthetic electron transport and the scavenging of H$_2$O$_2$ does not dissipate ΔpH across the thylakoid membrane for ATP regeneration, but rather, it promotes the formation of ΔpH. That is, RISE can only be relieved by the oxidation of PQH$_2$. Conversely, RISE can be induced only by the reduction of PQ to oxidize P700 in PSI.

**CONCLUSION**

In the present research, we showed that RISE functioned on the donor side of PSI to oxidize P700 in wild type cyanobacterium, _S. elongatus_. In phase β, P700 in PSI is oxidized in response to suppressed photosynthetic CO$_2$ assimilation (Figure 2C; Shaku et al., 2016; Shimakawa et al., 2016b). The oxidation of P700 is driven by two mechanisms: (1) acidification of luminal side of the thylakoid membrane (i.e., ΔpH) lowers the oxidation activity of PQH$_2$ in Cyt b$_6$f (Trubitsin et al., 2003; Kramer et al., 2004); and (2) accumulation of PQH$_2$ suppresses the Q-cycle in Cyt b$_6$f to lower the oxidation activity of PQH$_2$ (i.e., RISE) (Shaku et al., 2016). Under low CO$_2$ in phase β, addition of NaHCO$_3$ stimulated LEF and caused the reduction of the NADPH pool (Figure 4). These results suggest that a donor-side limitation of electron flow in PSI arises, as shown in the oxidation of P700 (Shaku et al., 2016; Shimakawa et al., 2016b). Added NaHCO$_3$ relieves the donor-side limitation to enhance electron flux to oxidized P700, leading to NADPH production. Unfortunately, NaHCO$_3$-dependent acceleration of LEF cannot be considered conclusive evidence for RISE operating, because stimulated photosynthesis by NaHCO$_3$ not only oxidizes PQH$_2$ but also dissipates ΔpH. Thus, at this point, we could not exclude the possibility that a ΔpH-dependent control of electron flow from Cyt b$_6$f to oxidized P700 functions as depicted in Figure 4. We therefore continued to determine whether the H$_2$O$_2$ scavenging reaction stimulated reduction of NADP$^+$, in order to elucidate the mechanism of suppressed PQH$_2$ oxidation. Some peroxidases, including thioredoxin peroxidase and peroxiredoxin Q, require LCF-supplied NADPH as the electron donor for continuous scavenging of H$_2$O$_2$ (Yamamoto et al., 1999; Miyake and Asada, 2003; Stork et al., 2009). In other words, the H$_2$O$_2$ scavenging reaction by peroxidases drives LCF with the formation of ΔpH (Schreiber and Neubauer, 1990; Yamamoto et al., 1999; Miyake and Asada, 2003; Stork et al., 2009). The reduction of NADP$^+$ was enhanced by electron flux through LCF upon addition of H$_2$O$_2$ to _S. elongatus_ cells in phase β (Figure 5); concomitantly, oxidation of PQH$_2$ enhanced electron flux to NADP$^+$, which strongly supports the idea that RISE is regulated by the redox state of PQ, as reported by Shaku et al. (2016).

**AUTHOR CONTRIBUTIONS**

CM conceived the original screening and research plans and supervised the experiments. GS and KS performed all the
experiments. CM, GS, and KS conceived the project, designed the experiments, analyzed the data, and wrote the article.

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