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SHORT COMMUNICATION

Relative contributions of PGR5- and NDH-dependent photosystem I cyclic electron flow in the generation of a proton gradient in Arabidopsis chloroplasts

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Abstract

Main conclusion **Respective contributions of PGR5- and NDH-dependent cyclic electron flows around photosystem I for generating the proton gradient across the thylakoid membrane are ~30% and ~5%.**

The proton concentration gradient across the thylakoid membrane (ΔpH) produced by photosynthetic electron transport is the driving force of ATP synthesis and non-photochemical quenching. Two types of electron transfer contribute to ΔpH formation: linear electron flow (LEF), and cyclic electron flow (CEF, divided into PGR5- and NDH-dependent pathways). However, the respective contributions of LEF and CEF to ΔpH formation are largely unknown. We employed fluorescence quenching analysis with the pH indicator 9-aminoacridine to directly monitor ΔpH formation in isolated chloroplasts of Arabidopsis mutants lacking PGR5- and/or NDH-dependent CEF. The results indicate that ΔpH formation is mostly due to LEF, with the contributions of PGR5- and NDH-dependent CEF estimated as only ~30% and ~5%, respectively.

Keywords: Arabidopsis; Cyclic electron transfer; non-photochemical quenching; PGR5; Photosynthesis; ΔpH

Abbreviations

CEF, cyclic electron flow

ΔpH, proton concentration gradient across the inside and outside of the thylakoid membrane

Δψ, membrane potential

LEF, linear electron flow

NDH, NAD(P)H-dehydrogenase

NPQ, non-photochemical quenching

PGR5, proton gradient regulation 5

PMF, proton motive force

PSI, photosystem I

PSII, photosystem II

9-AA, 9-aminoacridine.

Introduction

Photosynthesis, which is carried out in chloroplasts, sustains almost all life through converting sunlight energy to chemical energy. The chloroplast interior is compartmentalized by the thylakoid membrane, which is critical for the photochemical reaction. The inside of the thylakoid membrane, called the lumen, is where water oxidation occurs during photosynthesis. On the other side of the thylakoid membrane (the stroma), carbon dioxide is assimilated. During photosynthesis in chloroplasts, electrons are transferred, in order, from photosystem II (PSII), plastoquinone, cytochrome b_6f complex, plastocyanin, photosystem I (PSI), and ferredoxin. Reduced ferredoxin potentiates the activity of the ferredoxin-$NADP^+$ oxidoreductase to reduce $NADP^+$ to produce NADPH. Upon water oxidation in PSII as well as the photosynthetic electron transfer through the cytochrome b_6f complex, H^+ accumulates on the luminal side, resulting in the generation of a proton gradient across the thylakoid membrane (ΔpH). ΔpH is then used for ATP synthesis. Synthesized NADPH and ATP are utilized to fix carbon dioxide in the Calvin-Benson cycle (Blankenship 2002).

In addition to ATP synthesis, several physiological features of ΔpH formation are known. For example, ΔpH greatly contributes to the high-light stress response in plants (Ruban 2016). When plants are exposed to intense light, excessive light energy produces reactive oxygen species, which damage the photosynthetic apparatus as well as other cellular components (Kaushik and Roychoudhury 2014; Yamori 2016). Thus, plants must utilize various stress-response mechanisms to protect cells from light stress. Non-photochemical quenching (NPQ), which dissipates absorbed light energy as heat, is a well-known mechanism to avoid high-light stress (Ruban 2016). NPQ consists of separate components such as pH-regulated

energy dissipation in the PSII antenna protein (qE), state transitions, and photoinhibition. qE is a major component of NPQ in land plants, and it is regulated by zeaxanthin content and ΔpH (Kalituho et al. 2007; Ruban 2016). Under low-intensity light, zeaxanthin is converted into violaxanthin by zeaxanthin epoxidase. Violaxanthin cannot efficiently dissipate energy as heat (Kaňa et al. 2016). Thus, under intense light, violaxanthin is converted into zeaxanthin by violaxanthin de-epoxidase, whose activity is enhanced by acidification of the thylakoid lumen, which is accelerated by photosynthetic electron transport (Niyogi et al. 1998). A mutation in *NPQ4*, which encodes the PSII subunit PsbS, abnormally decreases qE, indicating that NPQ4/PsbS contributes to qE induction (Li et al. 2000). NPQ4/PsbS-dependent qE induction is also regulated by ΔpH across the thylakoid membrane (Li et al. 2004; Johnson and Ruban 2011). These mechanisms can dissipate absorbed light energy, and thus the control of qE depends on the formation of ΔpH across the thylakoid membrane, which is critical for plants to control photosynthesis under different light conditions.

Cyclic electron flow (CEF) returns electrons from PSI to plastoquinone, which contributes to the formation of ΔpH (Shikanai 2007). In higher plants, two CEF pathways are known: the proton gradient regulation 5 (PGR5)-dependent pathway, and the NAD(P)H dehydrogenase (NDH)-dependent pathway (Shikanai 2016; Shikanai and Yamamoto 2017). However, the contributions of the respective electron transfer pathways to ΔpH generation have not been definitively determined. Here, to calculate the contribution of each CEF type to ΔpH formation, we employed fluorescence quenching analysis with the pH indicator 9-aminoacridine to directly monitor ΔpH formation in isolated chloroplasts of Arabidopsis mutants lacking PGR5- and/or NDH-dependent CEF. The results indicate that ΔpH formation is mostly due to LEF, with

the contributions of PGR5- and NDH-dependent CEF estimated as only ~30% and ~5%, respectively.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana Ecotype Columbia-0 was used as the wild-type (WT). The PGR5 mutant (*pgr5*) (Munekage et al. 2002), NDH mutant (*crr4-2*) (Kotera et al. 2005), and PGR5 overexpression line (35S:PGR5#2) (Okegawa et al. 2007) were kindly provided by Professor Toshiharu Shikanai of Kyoto University. Plants were grown on half-concentration Murashige and Skoog medium. After treatment at 4°C in the dark for 3 to 5 days, the plants were grown at 23°C with continuous light of intensity 50 μmol photons m^{-2} s^{-1}. After 14 days, plants were transferred to soil and further grown for 1 to 2 months under short-day conditions (8 h light with intensity 200 μmol photons m^{-2} s^{-1} at 23°C / 16 h dark at 16°C).

Chlorophyll fluorescence measurement

Chlorophyll fluorescence of intact leaves was measured using a Dual-PAM-100 (Walz). The minimum chlorophyll fluorescence at the open PSII center (*Fo*) was determined by measuring A_{655} under a light intensity of 0.05–0.15 μmol photons m^{-2} s^{-1}. A saturating pulse of white light (400 ms) was applied to determine the maximum chlorophyll fluorescence at the closed PSII centers when in the dark (*Fm*) and during actinic light illumination (*Fm*'). Steady-state chlorophyll fluorescence (*Fs*) was measured after 5 min of actinic light illumination. NPQ was calculated as (*Fm* – *Fm*')/*Fm*'. Plants were dark-adapted for 15 min before measurement.

ΔpH measurement using 9-aminoacridine (9-AA)

Leaves (5 g) were collected from the plants grown in soil, and the leaves were disrupted with a homogenizer (HG30, Hitachi) in a buffer containing 330 mM sorbitol and 5 mM $MgCl_2$, then filtered through Miracloth (Calbiochem). The filtered sample was centrifuged at 4,000 × *g* for 7 min using a cooling centrifuge (CR 20 G, Hitachi), and the pellet was resuspended in a buffer containing 330 mM sorbitol, 10 mM KCl, 5 mM $MgCl_2$, and 50 mM HEPES-KOH (pH 7.6). Intact chloroplasts were isolated from the resuspension by Percoll density gradient centrifugation (Seigneurin-Berny et al. 2008). The chloroplast suspension was collected by centrifugation for 10 min at 4,000× *g*, and the sample was resuspended in a buffer containing 0.45 □M sorbitol, 20 mM sodium citrate, 10 mM EDTA, 10 mM $NaHCO_3$, 0.1% bovine serum albumin, 5 mM $MgCl_2$, and 20 mM HEPES/MES (pH 8.0) and used for measurements.

Intactness of the isolated chloroplasts were checked by the Hill-reaction as described previously (Heber and Santarius 1970). Briefly, isolated chloroplasts (7 μg chlorophyll ml^{-1}) were mixed with 0.5 mM potassium ferricyanide and 1 mM NH_4Cl. Photoreduction of ferricyanide was monitored by measuring absorbance change at 420 nm under white-light illumination provided by a halogen ramp.

We used 9-AA fluorescence as a pH indicator to monitor ΔpH across the thylakoid membrane (Schuldiner et al. 1972). Briefly, 1 μM 9-AA (final concentration) was added to the suspension of intact chloroplasts (equivalent to 35 μM chlorophyll), and the fluorescence intensity was measured using the Dual-PAM-100 connected with Dual-ENADPH and Dual-DNADPH modules (Walz). Excitation was done with a 365-nm LED, and fluorescence

emission was detected between 420 and 580 nm. ΔpH values were calculated as follows.

The concentration of 9-AA in the lumen, $[A]_{T,in}$, is expressed as:

$$[A]_{T,in} = [A]_{T,init} Q \times V_{in} / V_{out} \quad (1)$$

where $[A]_{T,init}$ is the initial concentration of 9-AA, Q is the attenuation rate of the fluorescence intensity of 9-AA, V_{in} is the volume of the lumen, and V_{out} is the sample volume (1.4 ml). V_{in} is set as 56 µl mg^{-1} chlorophyll (Johnson et al. 2012). Because the chlorophyll concentration was 35 µM for all measurements, V_{in} could be calculated as $0.056 \times (0.035 \times 1.4) = 2.74 \times 10^{-3}$ ml. The concentration of 9-AA in the stroma, $[A]_{T,out}$, is expressed as:

$$[A]_{T,out} = [A]_{T,init} \times (1 - Q) \quad (2)$$

and the ratio of the internal and external concentrations is expressed as:

$$[A]_{T,in} / [A]_{T,out} = [Q / (1 - Q)] \times V_{in}/V_{out}. \quad (3)$$

Because 9-AA is a diamine and can move freely inside and outside the membrane in the uncharged state, the ratio is also expressed as:

$$\frac{[A]_{T,in}}{[A]_{T,out}} = \frac{[A]_{in} + [AH^+]_{in} + [AH_2^{++}]_{in}}{[A]_{out} + [AH^+]_{out} + [AH_2^+]_{out}}$$

$$= \frac{K_1K_2 + K_1[H^+]_{in} + [H^+]_{in}^2}{K_1K_2 + K_1[H^+]_{out} + [H^+]_{out}^2}$$

where $K_1 = [AH^+][H^+] / [AH_2^{++}]$, $K_2 = [A][H^+] / [AH^{++}]$, and $[A]_{in} = [A]_{out}$. Because pK_1 and pK_2 of 9-AA are »pH_{in} and «pH_{out}, respectively (or « $[H^+]_{in}$ and » $[H^+]_{out}$, respectively), ΔpH could be expressed as:

$$\Delta pH = \log ([H^+]_{in} / [H^+]_{out}) = \log([A]_{T,in} / [A]_{T,out}) = \log([Q / (1 - Q)] \times V_{in} / V_{out}). \quad (4)$$

Results and discussion

To investigate how CEF contributes to the formation of ΔpH, we measured pH values in the lumen and the stroma using 9-AA fluorometry. At neutral pH, 9-AA is uncharged and therefore has high membrane permeability. Once it is protonated in weakly acidic solution, however, its membrane permeability is markedly reduced. The important point is that when 9-AA is protonated, the fluorescence yield is greatly reduced. Using this property, a system for measuring the proton concentration gradient across a membrane was established (Schuldiner et al. 1972). Specifically, when 9-AA is mixed with isolated chloroplasts and incubated in the dark, 9-AA in the non-protonated state can freely move back and forth between the stroma and the lumen, so the abundance of the molecule is almost the same across the membrane. Upon irradiation to drive photosynthesis, however, the pH on the lumen side decreases, resulting in protonation of 9-AA. Then, because the membrane permeability decreases, 9-AA remains in the lumen and the quantum yield of fluorescence decreases. By monitoring the fluorescence intensity of the chloroplast suspension in this manner, the ΔpH across the membrane can be estimated. Figure 1 shows the typical fluorescence kinetics of the measurement. When irradiation with actinic light was used to drive photosynthesis, the fluorescence intensity of the chloroplast suspension dropped sharply and then reached a steady state. The decrease in fluorescence was reversed when irradiation was halted. The termination of the reduction of the fluorescence intensity after 5 min of illumination was defined as Q, which was used to calculate ΔpH (see Materials and Methods for details). We checked stability of intactness of the isolated chloroplasts by the Hill-reaction with ferricyanide used as an electron donor (Heber and Santarius 1970). Specifically, because ferricyanide is unable to penetrate the chloroplast envelope, intact chloroplasts do not reduce ferricyanide upon light illumination. We confirmed that isolated

chloroplasts only very slowly photoreduced ferricyanide even after 6 hours incubation on ice (Supplementary Fig. S1). Thus, the ΔpH measurement with 9-AA were done within 6 hours after the chloroplast isolation.

Figure 2 shows the levels of ΔpH in the WT, PGR5-deficient mutant (*pgr5*), NDH-deficient mutant (*crr4-2*), and PGR5 overexpression line (35S::PGR5#2) at different actinic light intensities. The ΔpH formation in WT (blue) can be divided into three stages. The rise in the first stage occurred under weak light (<30 μmol photons m^{-2} s^{-1}), and the rise in the second stage occurred at approximately the intensity for plant growth (~150 μmol photons m^{-2} s^{-1}). When the actinic light intensity was greater than ~150 μmol photons m^{-2} s^{-1}, ΔpH formation slowly increased linearly with light intensity. The ΔpH formation in *pgr5* (red) was generally smaller than in WT (Fig. 2). In particular, at light intensities from 89 to 209 μmol photons m^{-2} s^{-1}, very little increase in ΔpH was observed. The formation of ΔpH in *crr4-2* (green) was similar to that in WT. In contrast, ΔpH in 35S::PGR5#2 (yellow) was larger than that in WT at all light intensities (Fig. 2). Based on results of the ΔpH formation kinetics, the contribution of the PGR5-dependent pathway for generating ΔpH was estimated to be 25–30%, whereas the contribution of the NDH-dependent pathway was relatively small (≤5%).

Next, we investigated the effect of light intensity on NPQ induction in intact leaves of WT, *pgr5*, *crr4-2*, and 35S::PGR5#2 (Fig. 3). NPQ in WT (blue) increased in two phases; a rapid increase of NPQ was seen until ~180 μmol photons m^{-2} s^{-1}, and it leveled off when the light intensity exceeded ~200 μmol photons m^{-2} s^{-1}. Similar NPQ induction kinetics was observed in *crr4-2* (green). On the other hand, *pgr5* showed significantly less NPQ induction at all light intensities (red). The 35S::PGR5#2 overexpression line produced greater NPQ induction than

WT at all light intensities (yellow). The induction kinetics of 35S::PGR5#2 were generally similar to those of WT, although a more rapid increase was observed at low-intensity light (<31 μmol photons m^{-2} s^{-1}). The properties of the NPQ induction in *pgr5* and 35S::PGR5#2 were essentially the same as those reported previously (Munekage et al. 2002; Okegawa et al. 2007).

Given that NPQ induction was not observed normally in *pgr5*, especially above 50 μmol photons m^{-2} s^{-1}, the rapid induction of NPQ in WT is likely due to ΔpH formation by the PGR5-dependent CEF. Recently, Wang et al. (2015) performed an electrochromic shift analysis to estimate the significance of CEF for generating proton motive force (PMF), which is composed of ΔpH and the membrane potential (Δψ). The results indicated that PMF in WT and *pgr5* are almost the same under low light (<46 μmol photons m^{-2} s^{-1}), whereas a ~40% reduction was observed in *pgr5* when light intensity was >138 μmol photons m^{-2} s^{-1} (Wang et al. 2015). Together with the results of our present study, these data suggest that, under low light intensity (<50 μmol photons m^{-2} s^{-1}), LEF predominates to generate ΔpH and PMF, and PGR5-dependent CEF becomes important when light intensity is >100 μmol photons m^{-2} s^{-1}. We observed a relatively steady-state ΔpH formation in *pgr5* at light intensities of 150 to 300 μmol photons m^{-2} s^{-1} (Fig. 2, red); however, such kinetics were not observed in the PMF generation as examined by electrochromic shift analysis (Wang et al. 2015). One possible explanation for this difference is that Δψ predominates at low light intensity, whereas when light intensity is increased above ~150 μmol photons m^{-2} s^{-1}, ΔpH formation by the PGR5-dependent CEF predominates for generating PMF. This hypothesis is further supported by a recent study by Yamamoto et al. (Yamamoto et al. 2016). Specifically, the authors estimated the respective contributions of ΔpH and Δψ for PMF generated at different light intensities, which showed that the contribution of ΔpH formation by

the PGR5-dependent CEF in PMF generation increases as light intensity increases above 134 μmol photons m^{-2} s^{-1}. In accordance with the increase in ΔpH formation, the contribution of Δψ for PMF generation was smaller in WT compared with the *pgr5* mutant. Notably, Δψ formation under moderate light intensity (~100 μmol photons m^{-2} s^{-1}) in *pgr5* was smaller than that in WT (Yamamoto et al. 2016; Shikanai and Yamamoto 2017), indicating that PGR5-dependent CEF is critical for Δψ formation to sustain PMF.

In conclusion, we directly measured the contribution of light intensity to ΔpH formation in WT and several CEF mutants using 9-AA fluorescence quenching, and we compared the results with those of previous electrochromic shift analyses. The results indicate that ΔpH formation is associated with NPQ induction, supporting the importance of luminal pH for inducing qE-dependent NPQ. We also showed that PGR5- and NDH-dependent CEFs contribute differently to PMF generation. Specifically, PGR5 is involved in both ΔpH and Δψ formation for PMF control, but NDH-dependent CEF has only a minor contribution to ΔpH formation. To modulate the balance between ΔpH and Δψ, plants control the transfer of ions such as K^+ and Mg^{2+} across the thylakoid membrane (Song et al. 2004; Carraretto et al. 2013; Kunz et al. 2014; Armbruster et al. 2016; Herdean et al. 2016; Dana et al. 2016; Wang et al. 2017). Clearly, further analysis with iron transporter and channel mutants will be important to clarify the mechanistic regulation of PMF for control of photosynthesis. Although almost a half century has been passed since 9-AA fluorescence quenching was developed (Schuldiner et al. 1972), the methodology is still useful for this purpose.

Author contribution statement RS and SM designed the research. All authors performed the

experiments and SM wrote the paper. All authors approved the manuscript.

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Figure legends

Fig. 1 Light-induced fluorescence quenching of 9-AA. The chloroplast suspension was mixed with 1 μM 9-AA, and the change of 9-AA fluorescence was monitored upon switching the actinic light (AL) on and off. The attenuation of the reduction of the fluorescence intensity was defined as Q, which reflects ΔpH formation (see Materials and Methods for details).

Fig. 2 Effect of light intensity on ΔpH in isolated chloroplasts of WT (blue line), *pgr5* (red line), *crr4-2* (green line), and 35S::PGR5#2 (yellow line), which was calculated from 9-AA fluorescence changes (see Materials and Methods for details). Values are mean ± standard deviation of three biological replicates.

Fig. 3 Effect of light intensity on NPQ in intact leaves of WT (blue), *pgr5* (red), *crr4-2* (green), and 35S::PGR5#2 (yellow). After adaptation to dark for 15 min, actinic light of varying intensity was applied to the plants, and NPQ values were calculated from chlorophyll fluorescence after 5 min for each measurement. Values are mean ± standard deviation of three biological replicates.

Figure 1

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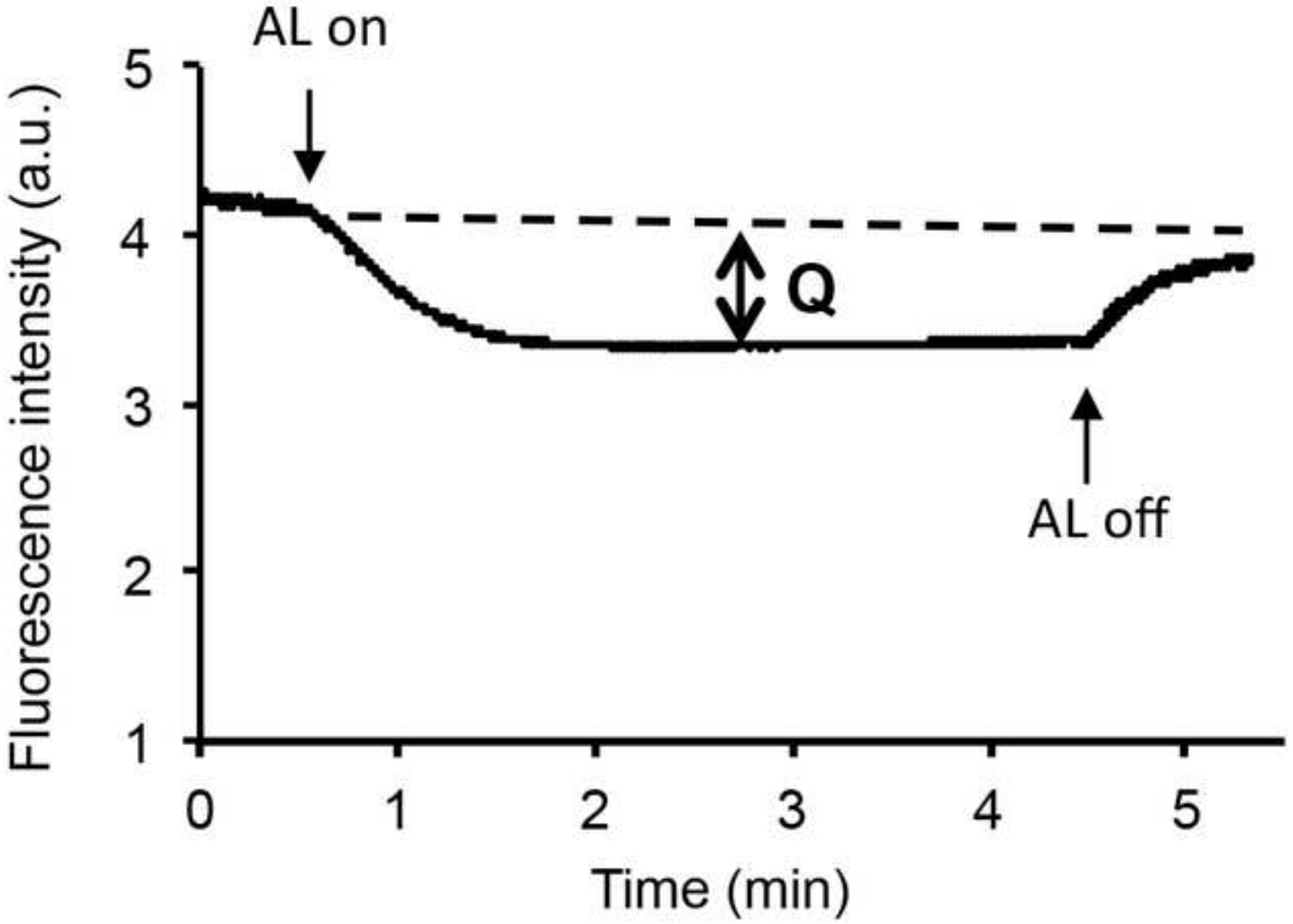

Figure 2

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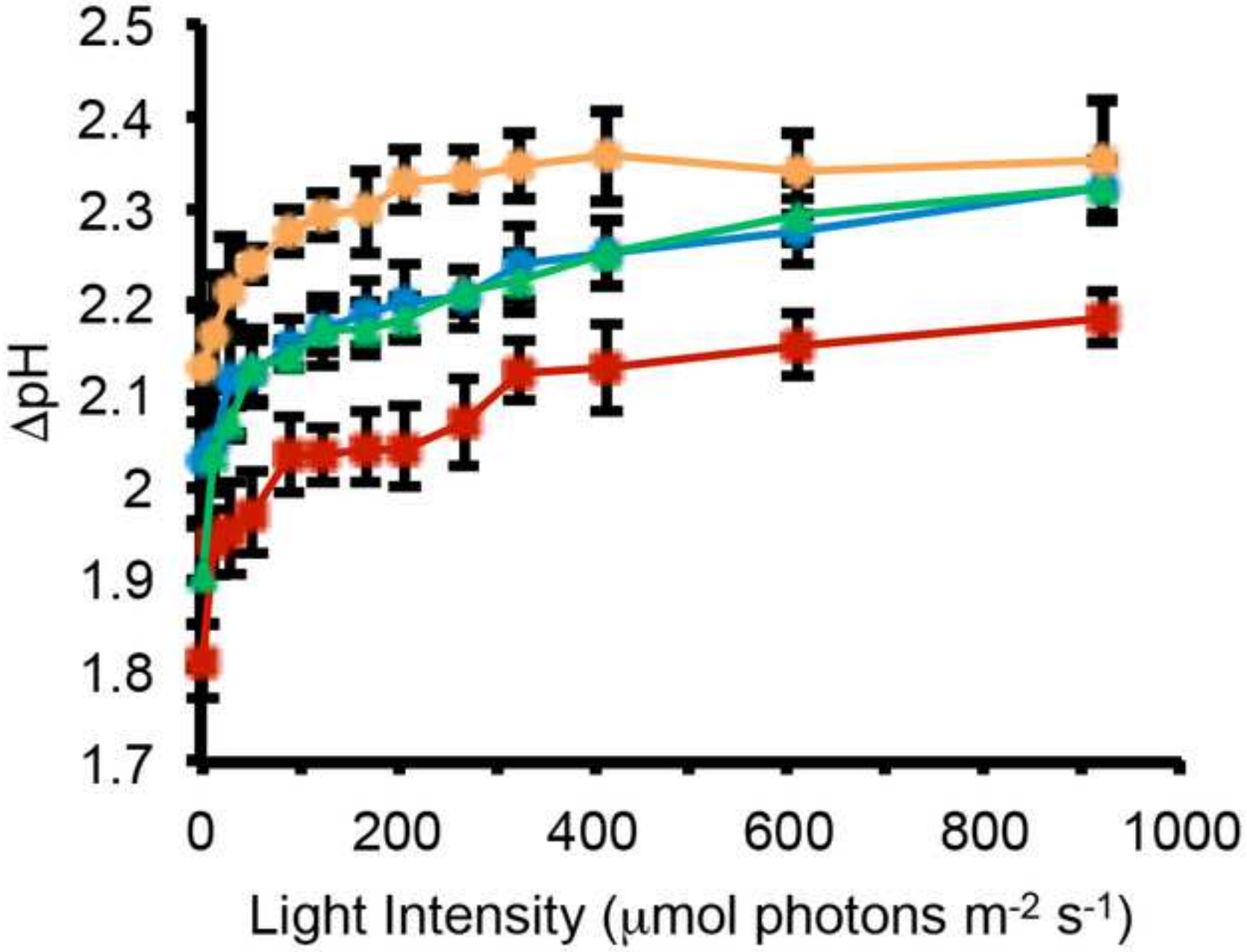

Figure 3

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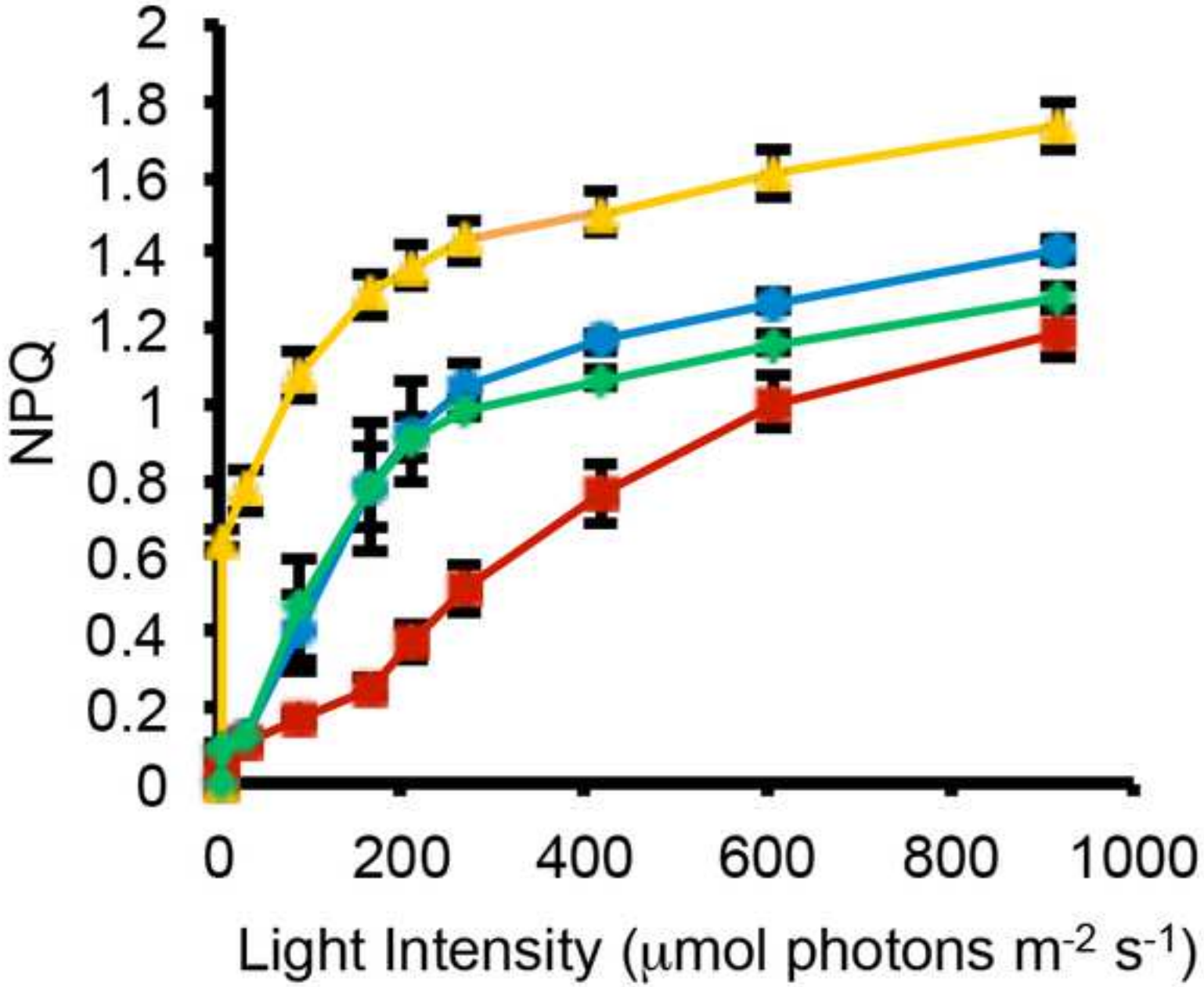

Supplementary Material Fig.S1