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Cyclic electron transport around photosystem I and its relationship to non-photochemical quenching in the unicellular green alga *Dunaliella salina* under nitrogen deficiency

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Abstract

Electron transport in photosystem II (PSII) and photosystem I (PSI) was estimated in terms of chlorophyll fluorescence and changes in P700 redox, respectively, in the unicellular green alga *Dunaliella salina* in the presence or absence of a nitrogen source in the culture medium. In a nitrogen-containing medium, the quantum yield of PSII (Φ_{II}) and that in PSI (Φ_I) were at the same level in low light, but cyclic electron transport around photosystem I (CET-PSI) was induced under high light as estimated from an increase in Φ_I / Φ_{II} . High light might further enhance the rate of electron transport in PSI by inducing the state 2 transition, in which the distribution of light energy is shifted to PSI at the expense of PSII. Nitrogen deficiency resulted in a decrease in Φ_{II} and an increase in Φ_I . As a consequence, the rate of CET-PSI was expected to increase. The high CET-PSI under N deficiency was probably associated

with a high level of energy quenching (qE) formation in PSII.

Key words:

Chlorophyll fluorescence, Cyclic electron transport, *Dunaliella salina*, Nitrogen deficiency, Nonphotochemical quenching, P700

Introduction

Stress is defined as an external factor that exerts an undesirable influence on plants, and it can cause various responses in an organism at all physiological levels. Plants have developed individual tolerance mechanisms to cope with environmental stresses during evolution, and these mechanisms vary greatly among different species and phylogenic groups. Under an environmental stress such as nitrogen deficiency, plants show ingenious ways of adaptation at all levels of organization, from the morphological to biochemical, molecular and physiological levels (Diaz et al. 2007). At biochemical and physiological levels, plants can cope with stress by altering photosynthetic electron transfer in various ways (Dijkman and Kroon 2002; Quiles 2006; Diaz et al. 2007; Ibanez et al. 2010).

Photons absorbed by chloroplasts drive photosynthetic linear electron transport in thylakoid membranes. Their energy is stored in the form of NADPH and ATP, which are required for photosynthetic carbon fixation. Regulatory processes that can adjust the efficiency of primary electron transport to the demands of stromal biochemical reactions are of considerable interest to photosynthesis researchers (Scheibe

and Stitt 1988; Foyer et al. 1990; Harbinson and Foyer 1991). Such regulation includes mechanisms that lead to a well-balanced distribution of energy between the two photosystems, to concerted optimization of the quantum yields between the two photosystems, and to an optimal levels of stromal enzyme activation that match the rates of ATP and NADPH production. Exposure of plants to stress has two opposite effects on the photosynthetic electron transport chain. First, photosystem I (PSI) is stimulated by stress (measured as the reduction rate of $P700^+$), which is related to enhanced reduction of the plastoquinone pool by either ferredoxin or NADPH (Havaux 1996; Bukhov et al. 1999). In contrast, the quantum yield of photosystem II (Φ_{II}) decreases so that the energy of absorbed photons is only partly used for carbon assimilation (Miyake et al. 2005a). When carbon fixation proceeds at a low rate, the excess photon energy damages PSII in the thylakoid membranes by stimulating the production of reactive oxygen species (Asada 1996,1999; Niyogi 2000). To minimize PSII photoinactivation, plant chloroplasts dissipate excess photon energy as heat, a protective mechanism that is known as nonphotochemical quenching (NPQ) of chlorophyll fluorescence (Demmig-Adams and Adams 1996; Niyogi et al. 1998; Müller et al. 2001).

NPQ of chlorophyll fluorescence is induced under high light conditions where Φ_{II} is low (Miyake et al. 2004, 2005a,b). The induction of NPQ requires a pH gradient across the thylakoid membrane. At present, two alternative pathways of electron transport have been proposed for the induction of NPQ in higher plants. The first is oxygen-dependent electron flow in chloroplasts, known as the water-water cycle (WWC; Asada 1999). The second proposed pathway is cyclic electron transport around PSI

(CET-PSI) (Heber and Walker 1992; Heber 2002; Makino et al. 2002). In CET-PSI, NADPH or ferredoxin, photoreduced at PSI, donates electrons to the cytochrome *b₆f* complex. Two pathways have been reported for CET-PSI. One is ferredoxin-dependent and is sensitive to Antimycin A, and the other is NAD(P)H -dependent and Antimycin A-insensitive (Mi et al. 1995, Endo et al. 1998), although there is a debate on the presence of other CET pathways (Miyake 2010). The physiological significance of CET-PSI is also a matter of debate. Munekage et al. (2004) demonstrated that CET-PSI is essential for efficient photosynthesis because it increases acidification of the thylakoid lumen, which triggers down regulation of PSII through the formation of NPQ, and prevents stromal over-reduction. Such NPQ formation by cyclic electron transport is also important for inducing thermal dissipation under excessive light conditions and for protecting PSI from irreversible photodamage.

In algae, chlororespiration is often coupled to NPQ of chlorophyll fluorescence. Therefore, in these organisms, the generation of proton gradient has been attributed to active chlororespiratory electron flow (Ting and Owens 1993; Jakob et al. 2001). Chlororespiration has been defined as a respiratory electron transport chain in the thylakoid membrane of chloroplasts involving on-photochemical reduction and plastoquinol oxidase activity (Bennoun 1982, 1994). Therefore, the effects of chlororespiration on NPQ formation, as well as CET-PSI, should also be considered when algal cells are used as an experimental material.

Recently, intensive studies on CET-PSI in algae, especially in *Chlamydomonas* has begun, and molecular components involved in CET-PSI have been proposed (Iwai et

al. 2010, Peltier et al. 2010, Alric 2010, Bonente et al. 2011). However, at present, information on quantitative flow analysis of CET-PSI is not enough.

Dunaliella is a unicellular green alga that can accumulate large amounts of β -carotene under different stress conditions (Ben-Amotz and Avron 1983). This economically important alga can survive in very high salt concentrations, thus it is considered as an interesting experimental material for stress physiology. In the present study, *D. salina* was used as a model system for examining the regulation of CET-PSI under nitrogen deficiency. In addition, we considered CET-PSI activity in response to the light intensity and its relationship to NPQ of chlorophyll fluorescence.

Materials and methods

Algal cultures and experimental treatments

Dunaliella salina Teod. UTEX 200 was obtained from The Culture Collection of Algae at the University of Texas at Austin. The cells were grown in a culture medium with 1.5M NaCl, as described by Shariati and Lilley (1994). Cultures were incubated in a culture room at 25°C and 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of light under a 16 h /8 h light/dark photoperiod with continuous shaking (100 rpm). When the cultures were at the exponential growth phase, they were harvested by centrifugation (3,000 x g for 5 min) and resuspended in fresh medium with or without nitrogen. For the measurement of chlorophyll fluorescence, changes in P700 redox and low temperature fluorescence spectra, the cells were collected by centrifugation (3,000 x g for 5 min) and resuspended in the supernatant (used medium) to adjust the cell density.

Cell enumeration and pigment analysis

The cell number was determined by light microscopy using a hemocytometer (Schoen 1988). For chlorophyll and β -Carotene extraction, an aliquot (1 ml) of alga cell suspension was precipitated by centrifugation at 10000 g for 5 min followed by the addition of 1 ml of 80% acetone (v/v) and , after vortexing, centrifuged at 10000 g for 2 min. The chlorophyll content was determined spectrophotometrically by the method of Arnon (1949). β -Carotene was assayed according to Ben-Amotz and Avron (1983). $E_{1\text{cm}}^{1\%}$ of 2273 at 480 nm was used to calculate the β -carotene concentration.

Chlorophyll fluorescence and P700

Chlorophyll fluorescence and changes in P700 redox were measured with a PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany). The emitter-detector units used for fluorescence and P700 measurements were ED 800T and ED 101, respectively. The cell suspension ($100 \mu\text{g}$ chlorophyll ml^{-1}) was pipetted into a glass cuvette connected to the light sources and the emitter-detector unit via fiberoptics. The principles of the saturation pulse method for the analysis of chlorophyll fluorescence quenching have been described by Schreiber et al. (1986). Φ_{II} was calculated as $(F_{\text{m}}' - F_{\text{s}})/F_{\text{m}}'$, q_{p} as $(F_{\text{m}}' - F_{\text{s}})/(F_{\text{m}}' - F_{\text{o}})$ and NPQ as $(F_{\text{m}} - F_{\text{m}}')/F_{\text{m}}'$.

The principle of P700 measurement was described by Klughammer and Schreiber (1994). Φ_{I} was calculated as $(P_{\text{m}}' - P_{\text{s}})/P_{\text{m}}$, where P_{m} represents the full P700^{+} population estimated under a saturating flash light (50 ms) by an XMT103 unit

under far-red light, Pm' is that under a saturating pulse under white actinic light, and P_s is that at steady-state $P700^+$ under white light. For Pm' measurements, cells were illuminated with far-red light just before and during application of the saturation pulse.

Low-temperature fluorescence spectra

Cell suspensions ($5\ \mu\text{g chlorophyll ml}^{-1}$) were preilluminated with low ($100\ \mu\text{mol m}^{-2}\text{s}^{-1}$) or high ($1500\ \mu\text{mol m}^{-2}\text{s}^{-1}$) levels of white light for 5 min, and spectra of chlorophyll fluorescence induced by excitation at 435 nm were then measured at -196°C with a spectrofluorometer (FP8500, Hitachi).

Results and discussion

Effects of N depletion on cell density, chlorophylls and carotene

The cells cultured in a medium containing 5 mM KNO_3 (N-containing medium) were harvested at the exponential phase and resuspended in a medium with or without an N source, after which cells were cultured under the same conditions for additional 96 h. In the N-containing medium, the cells showed very vigorous cell division in the first 24 h, and gradually reached the stationary phase of growth (Fig. 1a). At the same time, contents of β -carotene and chlorophylls increased. In contrast, in an N-deficient medium, the cells showed slow growth in the first 24 h (Fig. 1a), and no new synthesis of β -carotene or chlorophylls was observed (Fig. 1b,c). During this experiment, the chlorophyll a/b ratio remained at around 2.9, and did not change under N-deficiency (data not shown).

Measurements of Φ_{II} and Φ_I under N deficiency

The quantum yields of photosystem II (Φ_{II}) and photosystem I (Φ_I) were estimated by quenching analysis of chlorophyll fluorescence and changes in P700 redox, respectively (Fig. 2). Cells that had been cultured in the N-containing medium were harvested and resuspended in the medium with or without an N source (Fig. 2a, b), and cultured for additional 48 h in the respective media (Fig. 2c,d). As in tobacco (Miyake et al. 2004), Φ_{II} and Φ_I were both at the same level under low light immediately after the cells were transferred to either N-containing or N-deficient conditions (Fig. 2a, b). In contrast, Φ_I was considerably higher than Φ_{II} under high light in these conditions. High Φ_I / Φ_{II} in high light suggested that CET-PSI is induced in high light as in tobacco (Miyake et al. 2004). Further culture in the N-containing medium did not change the light response curves for Φ_{II} and Φ_I (Fig. 2c). However, N-deficiency for 48 h resulted in a significant enhancement of Φ_I and a decrease in Φ_{II} (Fig. 2d). A very high Φ_I / Φ_{II} value was achieved under high light in the N-deficient medium, suggesting that CET-PSI was significantly induced in this condition.

Effects of N depletion on the electron transport chain

The reduction levels of the primary electron acceptor Q_A , expressed as 1-qP, under low ($300 \mu\text{mol m}^{-2}\text{s}^{-1}$) and high ($1350 \mu\text{mol m}^{-2}\text{s}^{-1}$) light are shown in Fig. 3a. The reduction level was higher under high light. Notably, N-depletion (-N/48 in Fig. 3a) resulted in a higher level of Q_A reduction even under low light.

Levels of non-photochemical quenching (NPQ) in cells immediately after transferred to either N-containing or N-deficient conditions (cont/0 or -N/0 in Fig. 3b) or those in cells incubated in the N-containing medium for additional 48 h (cont/48 in Fig. 3b) were twice as high under high light as compared with those under low light. In these cells, Φ_I/Φ_{II} increased under high light (see Fig. 2a-c). The level of NPQ under high light in N-depleted cells (-N/48) was more than 4 times higher than that under low light, thus very high NPQ was associated with high Φ_I/Φ_{II} (see Fig. 2d). The positive correlation between NPQ and CET-PSI, as estimated from Φ_I/Φ_{II} (see Fig. 2), is consistent with the results in tobacco (Miyake et al. 2004). However, it is also clear that the level of NPQ is not the sole factor that determines Φ_I/Φ_{II} ratios. For example, NPQ formation in cells cultured in the N-containing medium for additional 48 h (cont/48 in Fig. 3b) showed relatively lower than that in control cells just after transfer (cont/0 in Fig. 3b) and, even though Φ_I/Φ_{II} ratios were comparable between two conditions. Probably, the additional culture for 48 h may proceed growth phase of cells from log phase to stationary phase (see Fig 1a), thus physiological conditions within cells might change. Another point, for which we need further elucidation, is the relatively high Φ_I/Φ_{II} under low light in N-depleted cells. In this condition, the cells showed almost comparable levels of NPQ with the N-containing culture. However, Φ_I/Φ_{II} ratio was much higher.

It has been reported that the ~~A~~ main portion of NPQ, energy quenching (qE), is triggered by the formation of transmembrane pH gradient that is formed when electrons path through the electron transport chains between the two photosystems. In

tobacco, CET-PSI was considered to be the main factor that induces NPQ via the additional formation of a transmembrane pH gradient (Heber and Walker 1992, Miyake et al. 2004, 2005a). The induction of NPQ might suppress Φ_{II} because a larger fraction of absorbed light energy in PSII was released as heat with an NPQ-associated dissipation mechanism (Miyake et al. 2005b).

However, it has been reported that, in algae, the generation of a proton gradient is associated with active chlororespiratory electron flow (Ting and Owens 1993; Jakob et al. 2001). Therefore, the effects of an inhibitor of plastid terminal oxidase (PTOX) n-propyl gallate on Φ_{II} , Φ_I and NPQ were examined using cells cultured in the N-containing medium (data not shown). No significant changes in Φ_{II} , Φ_I or NPQ were found with the addition of n-propyl gallate, suggesting that PTOX does not affect photosynthetic electron transport under low or high light in *Dunaliella*.

State transition under N deficiency

NPQ is induced by at least three independent mechanisms: the main portion of NPQ, called energy quenching (qE), is associated with regulatory thermal dissipation; beside qE, qT induced by the state 2 transition and qI induced by the photoinhibition of PSII account for certain proportions in NPQ (Quick and Stitt 1989).

In cyanobacteria, a relatively strong flow of chlororespiration induced the state 2 transition in the dark (Schreiber et al. 1995). Illumination of dark-adapted cells with weak actinic light or far-red light induced the state 1 transition and illumination with strong white light induced the state 2 transition, which reflected the redox state of

the plastoquinone pool. If the state transition takes place under light, the distribution of the illuminated light energy between the two photosystems might change. Therefore, to determine the transition state in N-deficient cells under high or low light, fluorescence spectra at -196°C were examined (Fig. 4).

The peaks of chlorophyll fluorescence at 685 and 695 nm were from PSII and the peak at around 710-730 nm was from PSI. The PSI peak was highest in N-deficient cells that were illuminated with high light. A similarly high PSI peak was found in cells that had been incubated for 48 h in the N-containing medium (control in Fig. 4) illuminated with high light, while the PSI peak was relatively low when the same cells were measured just after the transfer to new medium (0 h in Fig. 4). All samples illuminated with low light showed identical spectra, in which the peak for PSI was the lowest. These results showed that, in all cell samples examined, the transition to state 2 occurred when the illumination was changed from low light to high light. These results also indicate that N-depletion for 48 h did not change PSI/PSII ratios in the low light condition.

Discussion of cyclic electron transport around photosystem I (CET-PSI)

The rate of electron transport at PSII (J_{II}) and PSI (J_I) can be expressed as:

$$J_{II} = \Phi_{II} \times p \times d_{II} \times PAR$$

$$J_I = \Phi_I \times p \times d_I \times PAR$$

, where p represents the assumed absorption coefficient, and d_{II} and d_I are the distribution ratios of light energy to PSII and PSI, respectively (Miyake et al. 2005b),

i.e., $d_{II} + d_I = 1$.

Miyake et al. (2004) showed that illuminated light energy was distributed equally to both photosystem II and photosystem I in tobacco leaves.

In higher plants, p is generally considered to have a value of 0.84.

However, in an algal suspension, p varies depending on the cell density. Since we have to use a very dense cell suspension (100 μg chlorophyll/ml) to obtain a satisfactory S/N ratio in P700 measurement, we could not get reliable information on p values. When a thinner cell suspension is used, p values should be measured directly.

The difference between J_I and J_{II} was regarded as the flow rate of CET-PSI because linear electron flow at both photosystems must be the same (Miyake et al. 2004).

When we assumed that $d_{II} = d_I$, as in the case of tobacco (Miyake et al. 2004), the result that Φ_{II} and Φ_I were at the same level under low light in non-stressed conditions (Fig. 2a-c), suggesting that electron transport in PSII (J_{II}) and that in PSI (J_I) are at the same level, i.e., no significant CET-PSI was induced under low light. Furthermore, the induction of CET-PSI as estimated from an increase in Φ_I / Φ_{II} was evident only under high light, similar to the result in tobacco (Miyake et al. 2004). High light induced a state 2 transition, in which antenna of PSII migrate to PSI. As a result, d_I / d_{II} increased. Consequently, J_I / J_{II} increased because both Φ_I / Φ_{II} and d_I / d_{II} increased under high light.

Both N-deficient and control cells showed similar levels of the state 2 transition under high light. Total NPQ in N-deficient cells under high light, however,

was much greater than that in control cells (Fig. 3b), suggesting that an NPQ factor that is not qT (state transition), probably qE, was associated with high Φ_I / Φ_{II} in N-deficient cells. Consequently, very high Φ_I / Φ_{II} value, associated with high qE, and high d_I / d_{II} value associated with qT, might induce very high J_I / J_{II} value in N-deficient cells under high light.

It should be noted again, however, that all discussion of CET-PSI shown above is based on the assumption that $d_{II} = d_I$ in low light.

Nitrogen deficiency and CET-PSI

In this study, we do not show any clear evidence for a molecular mechanism that can explain high CET-PSI under N deficiency. Highly reduced electron transport chain (see Fig. 3a) and high NPQ (see Fig. 3b) were often observed under high light and low partial pressure of CO₂, and in these conditions CET-PSI was also high (Makino et al. 2002; et al. 2004, 2005a). Thus, a speculation is that N depression might result in a decrease in Rubisco content, the largest N reservoir in chloroplasts. This condition might induce accumulation of reducing power in stroma in a form of reduced ferredoxin or NADPH, both of which are considered to be mediators of CET-PSI.

Conclusion

The flow rate of CET-PSI, as estimated from Φ_I / Φ_{II} , increased under high light in *Dunaliella*, as in the case of tobacco. Furthermore, the state 2 transition under high light increased the distribution of light energy to PSI, which might induce higher

CET-PSI in *Dunaliella*, while the contribution of the state 2 transition in higher plants has been reported to be minor (Guadagno et al. 2010; Ishida et al. 2011). Nitrogen depletion induced very CET-PSI as estimated from very high Φ_I / Φ_{II} and high d_I / d_{II} under high light.

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Legends for figures

Fig. 1 Effects of N depletion on cell density, chlorophylls and β -carotene. Changes in the cell number (a), β -carotene content (b), and total chlorophyll concentration (c) under N-deficient stress. At time 0, cells in the exponential phase of growth were collected and resuspended in medium with or without an N source, and then cultured under the same conditions for 96 h. Values are the averages of three independent experiments, and the vertical bars represent the standard deviations.

Fig. 2 Light-intensity dependent changes in the quantum yield of photosystem II (Φ_{II}) and Photosystem I (Φ_I) in the cells cultured in N-containing medium (a, c) and N-depleted medium (b, d), just after transferring to fresh medium (a, b) and after additional 48-h incubation (c, d). Values are the averages of three independent experiments, and the vertical bars represent the standard deviations.

Fig. 3 Effects of N depletion on the electron transport chain. The level of Q_A reduction expressed as 1-qP (a) and NPQ of chlorophyll fluorescence (b) under low ($300 \mu\text{mol m}^{-2}\text{s}^{-1}$) or high ($1350 \mu\text{mol m}^{-2}\text{s}^{-1}$) light at time 0 (just after transfer to the new medium) and 48 h are shown. Values are the averages of three independent experiments, and the vertical bars represent the standard deviations.

Fig. 4 Effects of N depletion on low-temperature chlorophyll fluorescence spectra.

Cells were preilluminated for 5 min under low ($100 \mu\text{mol m}^{-2}\text{s}^{-1}$) or high ($1500 \mu\text{mol m}^{-2}\text{s}^{-1}$) white light. Each spectrum was the average of 5 repeated measurements.

Fluorescence intensity was normalized at 695 nm. All cell samples that were preilluminated under low light showed identical spectra regardless of their culture conditions. Therefore, a spectrum for the 0 hr control in N-containing medium is shown as reference.

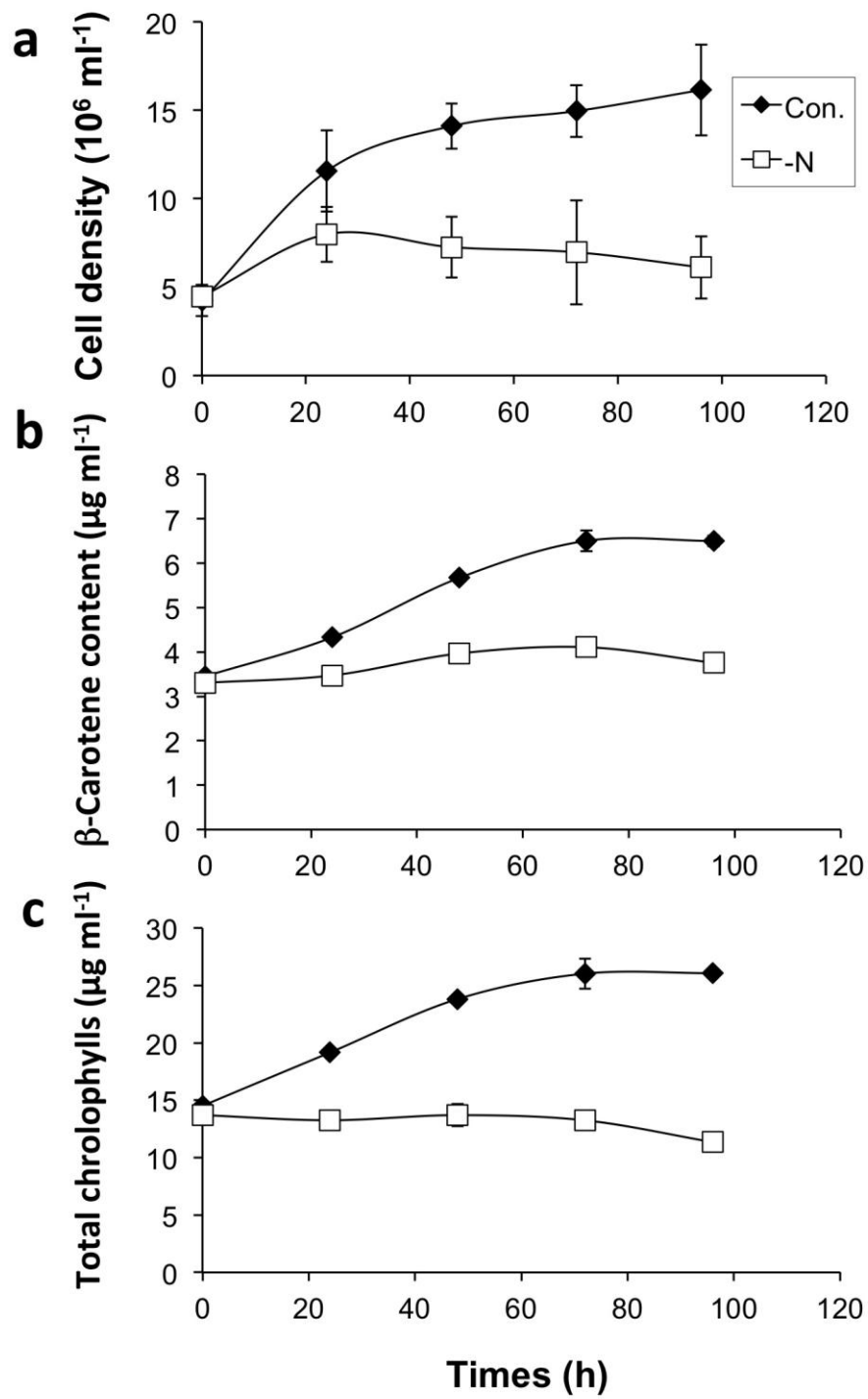


Fig. 1

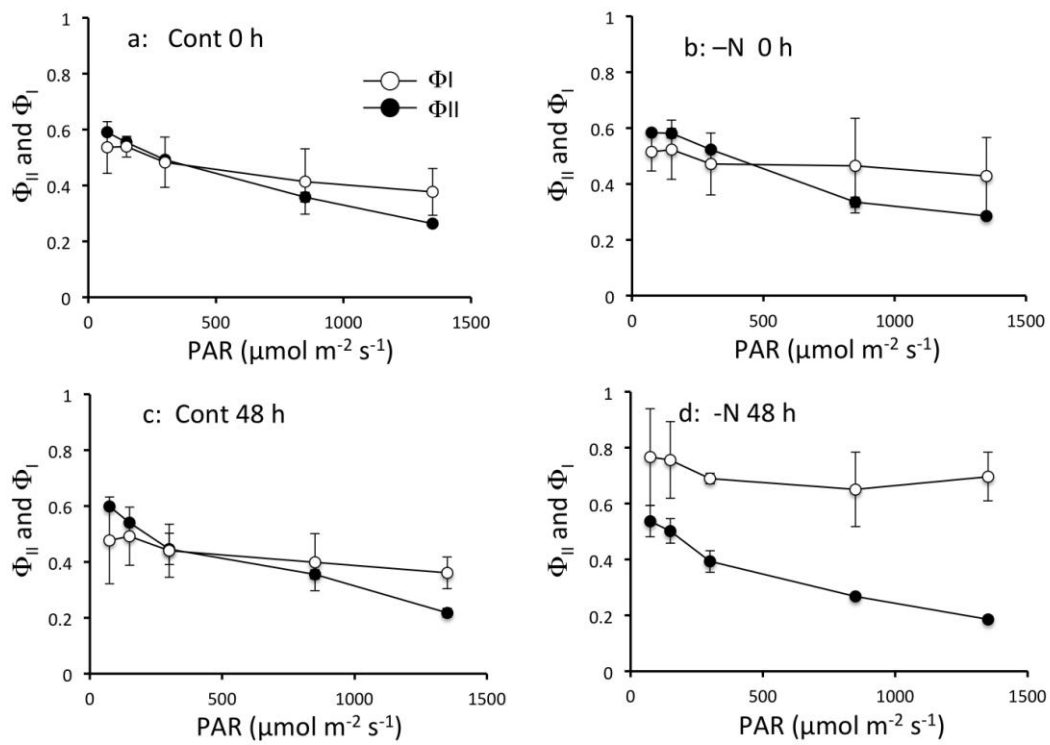


Fig. 2

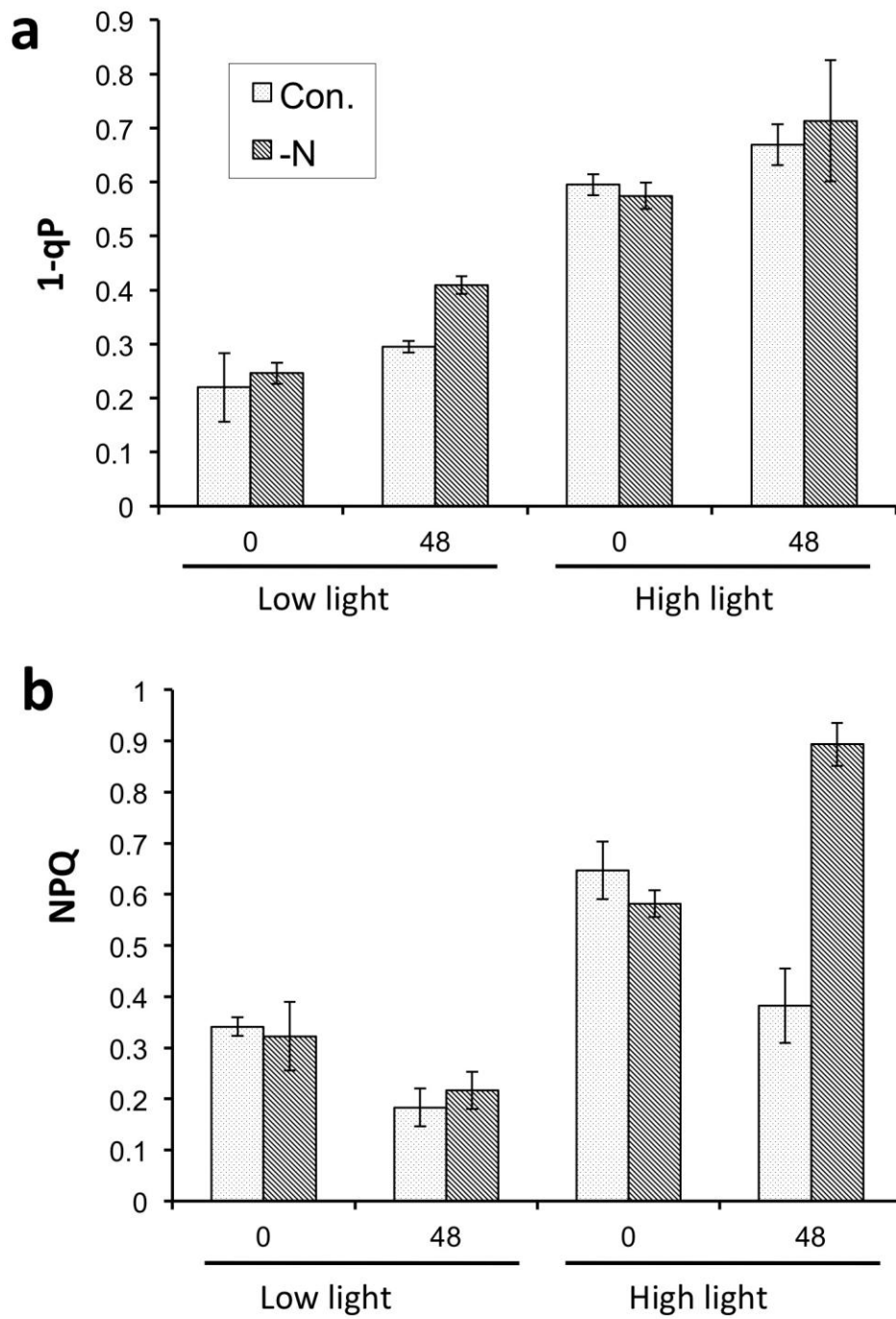


Fig. 3

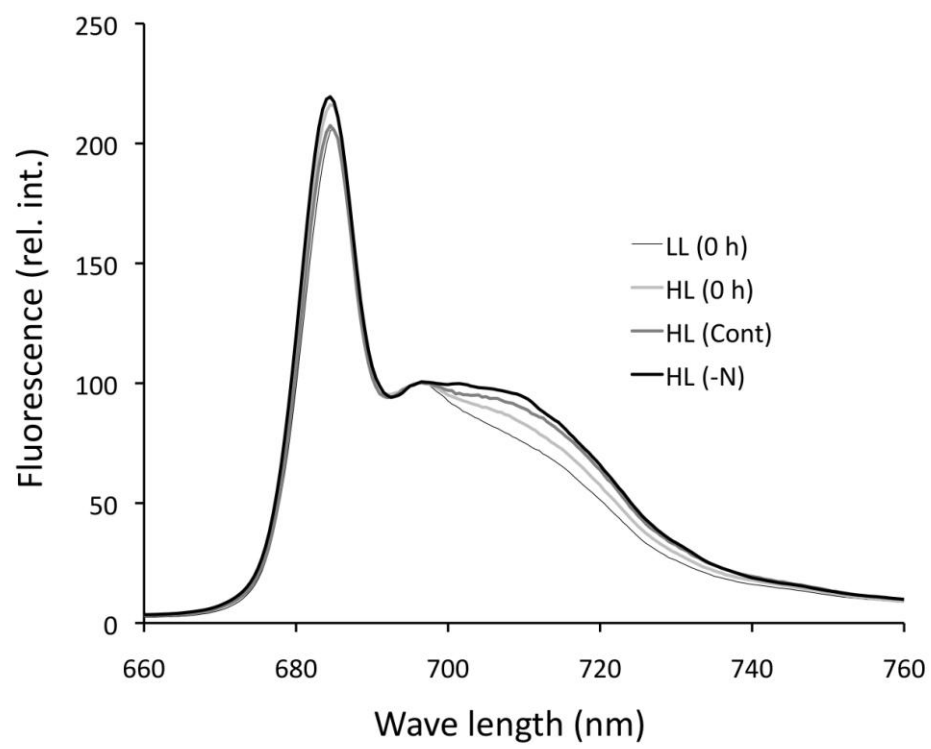


Fig. 4