Thiol modulation of the chloroplast ATP synthase is dependent on the energization of thylakoid membranes

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Thiol modulation of the chloroplast ATP synthase dependent on the membrane energization of thylakoid membranes

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ABSTRACT

Thiol modulation of the chloroplast ATP synthase γ subunit has been recognized as an important regulatory system for the activation of ATP hydrolysis activity (Mills, J. D., Mitchell, P. & Schurmann, P. (1980) *FEBS Lett.* **112**: 173-177), though the physiological significance of this regulation system remains poorly characterized. Since the membrane potential this enzyme requires to initiate ATP synthesis for the reduced enzyme is lower than that needed for the oxidized form (Junesch, U. & Gräber, P. (1987) *Biochim. Biophys. Acta* **893**: 275-288), the reduction of this enzyme was interpreted as effective regulation for efficient photophosphorylation. However, no concrete evidence has been obtained to date relating to the timing and mode of chloroplast ATP synthase reduction and oxidation in green plants. Thorough analysis of the redox state of regulatory cysteines of chloroplast ATP synthase γ subunit in intact chloroplasts and in leaves showed that thiol modulation of this enzyme is pivotal in prohibiting futile ATP hydrolysis activity in the dark, but the physiological importance of efficient ATP synthesis driven by the reduced enzyme in the light was not demonstrated. In addition, we investigated the significance of the electrochemical proton gradient in reducing the γ subunit by the reduced form thioredoxin in chloroplasts, providing strong insights into the molecular mechanisms underlying the formation and reduction of the disulfide bond on the γ subunit *in vivo*.

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INTRODUCTION

Chloroplast ATP synthase (CF_oCF_1) is known as a thiol enzyme; a disulfide bond located on the γ subunit is reduced by reducing equivalents produced by the photosynthetic electron transfer reaction. The key protein mediating the transfer of the reducing equivalents from photosynthetic reactions to the disulfide bond on the target enzyme is thioredoxin (Trx) in the chloroplast stroma, originally identified in *Escherichia coli* as a ribonucleotide reductase cofactor in 1964 (1). The significance of Trx in chloroplasts as a reducing equivalent mediator was first characterized in 1977 (2), and the basic concept of thiol modulation of chloroplast enzymes by the reduced form Trx was established by Buchanan et al. (3). The catalytic moiety of the chloroplast ATP synthase, CF_1, is a target of thiol modulation, and the role of Trx in activation of the ATP hydrolysis activity of the isolated CF_1 was demonstrated in the presence of dithiothreitol (4). The γ subunit of spinach CF_1, possessing four cysteines in its amino acid sequence, is critical for this thiol modulation. Two of these cysteines (Cys199 and Cys205) are located in the central region of the subunit and have been shown to be important for redox regulation (5-6). Interestingly, the region containing the two regulatory cysteines, the so-called ‘inserted sequence’, is comprised of a total of about 40 amino acid residues, and is only present in the γ subunit of oxygen evolving photosynthetic organisms. Comparison of whole sequences of the γ subunit from various organisms shows that the γ subunit of cyanobacteria displays only 30 amino acids within this insertion and lacks the two regulatory cysteines (7-8).

In the past two decades research has been primarily focused on the chemical

reduction of the disulfide bond located on the γ subunit of the isolated CF_1-ATPase, and the relationship between the reduction of the disulfide bond and stimulation of the activity has been investigated in detail (9-12). The redox regulation of rotation of the γ subunit in the $\alpha_3\beta_3$ hexamer was then visualized using the chimeric $\alpha_3\beta_3\gamma$ complex obtained from thermophilic bacteria, whose γ subunit was designed to contain the regulatory region of the spinach γ subunit in the central domain (13-14), and more recently using the $\alpha_3\beta_3\gamma$ complex of thermophilic cyanobacteria containing the regulatory segment from spinach γ subunit (Kim et al, 2011). However, there have been no *in vivo* reports to date pertaining to the reduction of the disulfide bond on the γ subunit of CF_oCF_1 complex by reducing equivalents supplied by the photosynthetic electron transfer system alone, although photosynthetic activation of CF_oCF_1 was indirectly measured using the electrochromic shift of the carotenoid in thylakoid lumen (15-16). In addition, most studies concerning activation of the thiol enzymes by reduction have historically been carried out *in vitro* predominantly by way of the classic reducing agent dithiothreitol (DTT) in the millimolar range or, more recently, the substitute Tris(2-carboxyethyl)phosphine.

In this study, particular focus was placed on reduction of the γ subunit by the *'in vivo'* photosynthetic electron transport system, and reduction and reoxidation of the disulfide bond on the γ subunit in intact chloroplasts and in leaves was investigated. The combined methodologies lead to the elucidation of the physiological relevance of this key enzyme: reduction of the γ subunit of CF_oCF_1 for enzyme activity, and oxidation for the maintenance of photosynthesis as a regulatory mechanism. In addition, the conditions required for the effective reduction and oxidation of the γ subunit *in vivo* were studied.

RESULTS

Photoreduction of the chloroplast ATP synthase. We first sought to determine whether completely reduced or oxidized CF_1 γ subunit using standard chemical reduction or oxidation treatments could be visualized to a reasonable degree by our 4-acetoamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS) labeling method (Fig. S1A). Following AMS labeling, the redox state of the γ subunit was measured by differences in the mobility of labeled protein bands on SDS-PAGE caused by the difference in the number of incorporated AMS molecules. Since the signal intensities of the western blotting of the fully reduced and the oxidized forms γ subunit were obviously different each other due to the specificity of the polyclonal antibody applied to the experiments, the integrated band intensities using the same amounts of proteins were determined respectively and the averaged ratio 1:5.4 (reduced : oxidized) was determined. This ratio value was used for the calibration of the following studies. The photo-reduction level of the γ subunit was calculated as the ratio of the band intensities of the reduced form and that of the total γ subunits (reduced form plus oxidized form) shown in Fig. 1A. In contrast, the signal intensities of the reduced and the oxidized form FBPase were equivalent (Fig. S1B).

By using the method described above, the difference in the level of photoreduction of the γ subunit of CF_1 in intact chloroplasts under various light intensities was then studied (Fig. 1). Even at 12.5 or 25 μmol photons·m^{-2}·s^{-1}, photoreduction of the γ subunit was partially observed. At a light intensity of more than 800 μmol photons·m^{-2}·s^{-1}, the photoreduction level of the γ subunit was almost constant

and saturated at around 80-90 % of the total γ. The rate of reduction of the γ subunit after turning on the light was independent of the light intensity, and was somewhat slow. It reached the maximal reduction level within 3 min, though the rapid oxidation was observed immediately after turning off the light.

Effects of various inhibitors on the γ subunit reduction. In order to clarify the significant factors which determine the reduction level of the γ subunit, we investigated the effect of inhibitors of the photosynthetic electron transport and an uncoupler. For this purpose, DCMU, a specific inhibitor to block the electron flow from photosystem II to plastquinone, DBMIB, a specific inhibitor to block the interaction between plastquinone and cytochrome b_6-f, and the uncoupler, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were examined. After illumination of the chloroplasts with sufficient light intensity (800 μmol photons·m^{-2}·s^{-1}) for one min, these inhibitors were added to the chloroplast preparation under light conditions. When the electrochemical gradient of proton ($\Delta\tilde{\mu}_{H^+}$) was collapsed in thylakoids by addition of FCCP, the reduction process of γ terminated immediately and oxidation started. The oxidation of γ was, however, much slower than that observed following the transition from light to dark and the full oxidation after addition of FCCP in the light required about 10 min (Fig. 2A). The effects of the electron transfer inhibitors, DCMU and DBIMIB, were more pronounced and the γ subunit was immediately oxidized after addition of them (Fig. 2A).

The relevance of the redox state of the γ subunit with the activation and inactivation of ATP synthase was further confirmed by measurement of ATP hydrolysis activity (Fig. 2B). Interestingly, all the treatments examined completely inhibited ATP

hydrolysis activity, though their sites of action are totally different.

In contrast, reduction level of FBPase, the typical thiol enzyme in chloroplasts, showed very different results; though the rapid oxidation was induced by the addition of DBMIB, the uncoupler FCCP had no effect on the reduction level of FBPase (Fig. 3), indicating that transfer of reducing equivalents from the photosynthetic electron transport system to Trx was not inhibited by uncoupling. In contrast, the effect of DCMU was limited for the oxidation of FBPase, suggesting the partial contribution of the cyclic electron flow for the reduction of Trx in chloroplasts.

To investigate the relevance of the reduction of the γ subunit to ATP synthase activity in detail, ATP synthesis was measured under the following light intensities: 25 μmol photons·m^{-2}·s^{-1}, 85 μmol photons·m^{-2}·s^{-1}, and 800 μmol photons·m^{-2}·s^{-1} (Fig. 4), resulting in different reduction levels of the γ subunit (Fig. 1B). Interestingly, the chloroplast ATP concentration reached the same level under these different light intensities, indicating ATP synthesis was promoted in chloroplasts irrespective of the redox level of the γ subunit. However, ATP concentration in chloroplasts decreased to the baseline level immediately by addition of FCCP due to the collapse of $\Delta\tilde{\mu}_{H^+}$, which is required for ATP synthesis

Redox change in the γ subunit of CF_oCF_1 in field-grown spinach leaves. Finally, the day-light response of the γ subunit of CF_oCF_1 in field grown spinach leaves was investigated. Spinach plants grown in the outdoor planter for eight to nine weeks after germination were used for this experiment, and diurnal changes in redox levels of the thiol enzymes in leaves were monitored by the method as described in 'Materials and Methods' (Fig. 5). Reduction of thiol enzymes initiated immediately after sunrise

irrespective of the weather, and reached a maximal level of around 60 to 80% within 3 h. The light intensity required to reach the maximal level was about 100 to 150 μmol photons·m^{-2}·s^{-1} (0.6 to 1.0 × 10^4 lux by the luxmeter[1]) (Fig. 5). Even though the light intensity outside further increased during daytime, the reduction levels of the γ subunit and FBPase was almost at the plateau level and did not change significantly. Oxidation of these enzymes was observed in proportion to the decrease in light intensity, and were converted into the fully oxidized form by sunset. On this process oxidation of FBPase started apparently earlier than that of the γ subunit.

DISCUSSION

The basic concept of photosynthetic activation of thiol enzymes in chloroplasts has been established through a number of seminal pieces of research (see the reviews (17-18)). Historically, the chloroplast ATP synthase is a well-known thiol enzyme, whose ATP hydrolysis activity is regulated by the chloroplast Trx-*f* (19-20), and the relationship between the reduction of the disulfide bond on the γ subunit and activation of the enzyme activity has been studied in depth. In addition, Kramer *et al*. reported activation of the sunflower CF_oCF_1 in the light based on the measurement of electrochromic shifts in the leaf thylakoids, concluding that the catalytic activation of CF_oCF_1 is not rate limiting to the induction of carbon assimilation under field conditions during a natural dark-to-light transition at sunrise (16). This study represents the first effort to determine

[1]Since the sensor of the light meter is very sensitive to the direction of the light source and not applicable to the measurement in our experiments under the outside day-light conditions, light intensity was measured by the luxmeter in the following experiments.

the redox state of CF_oCF_1 *in vivo*, though the actual redox states of CF_1 could not be determined in their measurements. To date, no direct evidence exists relating to the photosynthetic reduction of the γ subunit of CF_1 in leaves of plants in the natural environment. To confirm this phenomenon, direct assessment of the reduction level of the γ subunit using the thiol modifier AMS and a specific antibody raised against the regulatory region of the γ subunit was adopted in this study.

When the photosynthetic reduction of γ in intact spinach chloroplasts was measured, the maximum reduction level of the γ subunit was estimated to be 80-90% though other thiol enzyme FBPase was reduced only 40-50% under the same conditions. Although the cause of the fluctuation in reduction level observed in our study is unclear yet, we finally found that near complete reduction of the γ subunit and FBPase in plant leaves was also accomplished when the spinach leaves in the planter were directly frozen by liquid nitrogen immediately after the sampling under sunlight.

The major function of γ subunit reduction was thought to be to lower the energetic threshold for the ATP synthesis reaction (21-23). However, our reduction experiments using intact chloroplasts clearly show that reduction of the γ subunit never occurs without $\Delta\tilde{\mu}_{H^+}$ (Fig. 2A), as suggested by previous indirect measurements reported by Kramer *et al.* (16). Since reduction did not require strong light intensity as reported, reduction of γ never acts as a rate-limiting step for ATP synthesis in this context (see Fig. 1). In addition, direct measurement of the ATP concentration in the intact chloroplasts suggested that ATP synthesis itself initiated even under the insufficient light conditions for the full reduction of the γ subunit, and reached a significant level within a few minutes (Fig. 4). Note that the relevance between ATP synthesis activity and the reduction level of the γ subunit should be further studied since

the net ATP level we measured is the sum of the synthesis and the consumption of ATP in chloroplasts. Nevertheless, the result obtained here again suggests that the reduction of the γ subunit in chloroplasts is not a prerequisite for efficient ATP synthesis in the light. This conclusion corroborates previous reports that the oxidized ATP synthase can become active and support high rates of ATP synthesis even in the oxidized state (22, 24), though Wu *et al*. recently reported that the *cfq* mutant whose γ subunit was more difficult to be reduced by Trx showed a lower efficiency of ATP formation (25-26). This *cfq* mutant was originally obtained from the random mutagenesis study of *Arabidopsis thaliana*, when the mutant plants were selected that grew poorly under low irradiance but performed satisfactory at high irradiance (27). Consequently the resulted *cfq* mutant showed the phenotype that the γ subunit must be hardly reduced. The reduction level of γ returned to the basal level when FCCP was added in the light (Fig. 2). The slow oxidation observed might be due to the partial reducing effect of the reduced form Trx in chloroplasts, since the photosynthetic reduction of Trx occurs even in the presence of FCCP, as seen by photosynthetic reduction of FBPase (Fig. 3). As mentioned above, since $\Delta\tilde{\mu}_{H^+}$ formed by photosynthetic electron transport is a prerequisite for the reduction of γ (Fig. 2A), the gradual oxidation of γ observed in Fig. 2A implies that disulfide bond formation on the γ subunit occurs spontaneously in chloroplasts irrespective of the light/dark conditions and the reduced form Trx conclusively shifts the equilibrium between reduced and oxidized γ to the former. As suggested previously by the measurement of the change of the ATP hydrolysis activity using thylakoid membranes (28) and protoplasts (29), the observed tendency of the equilibrium shift to the oxidation state of CF_1 in chloroplasts is likely to confer the advantage of the ability to shut down inadequate ATP hydrolysis activity, thus avoiding futile ATP consumption

under conditions where photosynthesis does not occur.

Although the reduction level of thiol enzymes should be determined by the balance between the photosynthetic reduction and the spontaneous oxidation as mentioned, the factors involved into the oxidation process of thiol enzymes such as FBPase even at midday (Fig. 5), and the γ oxidation in the presence of FCCP (Fig. 2) remain unclear. From the study of the diurnal dynamics of photosynthesis in plants, midday depression of photosynthesis is a well-known phenomenon (30-32), and it would be important to study the potential higher order regulatory process for the thiol enzyme oxidation. One of the strong candidates involved in this oxidation process was proposed to be Trx itself from the study of redox change of the chloroplast ATP synthase (28). However, since slow oxidation of the γ subunit was observed in the presence of FCCP in the light, and FBPase was reduced even in the presence of FCCP (Fig. 3) as shown in this study, most of the Trx in the chloroplasts must be in the reduced form under the experimental conditions herein. Other oxidants and/or Trx as electorn acceptor(s) therefore can be postulated for oxidation of the γ subunit and thus the electron acceptor for the oxidation of thiol enzymes in chloroplasts in the dark should be subject to further investigation.

ATP synthase is now known as a motor enzyme that shows rotary motion during catalysis, with the γ subunit constituting the central axis subunit in the complex, rotating against the surrounding α and β hexagon. Reduction of the disulfide bond located on the γ subunit may therefore easily regulate rotation through a change in its conformation (13-14). Interestingly the region in the γ subunit responsible for redox-dependent conformational change is likely to be located near the subunit c, which forms the ring structure with 14 protomers (33) in the thylakoid membranes. Since the c

subunit contains the critical Glu residue for proton translocation during catalysis, it is feasible that the conformational change of the γ subunit or susceptibility of Trx to this subunit would be indirectly affected by the protonation of the Glu residues in the c-ring, or the conformational change of the lower part of γ itself by rotation (34-35). As the proteolysis of γ was affected by proton translocation via the c-ring (36), a certain conformational change of γ must be induced by protonation at the c subunit.

MATERIALS AND METHODS

Preparation of intact chloroplasts. Intact chloroplasts were isolated according to the method as described (37): 100 gram of market spinach leaves were homogenized for 10 sec in the mixer with 250 ml of the grinding solution containing 330 mM sorbitol, 5 mM sodium ascorbate, 0.05% (w/v) BSA, 2 mM EDTA, 50 mM HEPES-KOH (pH 7.6), 1 mM $MnCl_2$ and 1 mM $MgCl_2$. The homogenate was then filtered through four layers of gauze and centrifuged for 10 min at 2,000 × *g* at 4°C. The precipitate was suspended in grinding solution and intact chloroplasts isolated by centrifugation for 5 min at 4,500 × *g* with a discontinuous gradient of 40% (v/v) and 70% (v/v) Percoll in a solution containing 330 mM sorbitol, 5 mM sodium ascorbate, 50 mM HEPES-KOH (pH 7.6) and 1 mM $MgCl_2$. Intact chloroplasts were washed twice by centrifugation with wash buffer containing 330 mM sorbitol, 5 mM sodium ascorbate, 50 mM HEPES-KOH (pH 7.6) and 1 mM $MgCl_2$. The intact chloroplasts obtained were resuspended into the wash buffer at a chlorophyll concentration of 0.5 mg/ml and stored at 0°C.

Preparation of enzymes and antibodies. CF_1 was isolated from the broken

chloroplasts of spinach based on the method described in (38) with minor modifications. The broken chloroplasts were prepared by the standard procedure and washed five times with 10 mM sodium pyrophosphate to remove Rubisco. The chloroplasts were collected by centrifugation, suspended in 300 mM sucrose and 2 mM Tricine-NaOH (pH 8.0), and incubated for 10 min at 25°C. After centrifugation for 10 min at 15,000 × *g* at 4°C, the supernatant containing CF_1 was obtained and displayed a weak yellowish color. The purity of the resulted CF_1 fraction was normally more than 90%, estimated by Coomassie Brilliant Blue (CBB) staining after SDS-PAGE (Fig. S1), and sufficient to observe the redox states of the γ subunit by Western blotting (38).

For preparation of the specific antibody against the regulatory region of the γ subunit, the partial polypeptide of the CF_1-γ subunit comprised of ^{163}Ser to ^{249}Gln was expressed in *E. coli*. The expressed polypeptide was obtained from the cells by a French pressure cell (5501-M, Ohtake Works, Tokyo, Japan) at 4°C, and purified by successive chromatography with QAE Toyopearl column (Tosoh, Tokyo, Japan) with a 0 - 300 mM linear gradient of NaCl in 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 5 mM DTT and FPLC system with Hi-Load 75PG column (GE Healthcare, USA) eluted with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 200 mM NaCl, and 2 mM DTT. The purified polypeptide sample was then used as antigen to prepare the specific antibody.

The recombinant fructose 1,6-bisphosphatase (FBPase) of *Arabidopsis thaliana* chloroplasts lacking the signal sequence (^{59}Met to ^{417}Ala) was expressed in *E. coli*. The cells were collected by centrifugation and disrupted by sonication. The supernatant resulting from centrifugation at 125,000 × *g* for 30 min at 4°C was applied to a DEAE-Toyopearl (Tosoh, Tokyo , Japan) and eluted with a 0 to 300 mM linear gradient of NaCl in 25 mM Tris-HCl (pH 7.5). The peak fraction was collected and applied to

Butyl-Toyopearl column (Tosoh, Tokyo, Japan). The protein sample was eluted with a 30 to 0% reverse gradient ammonium sulfate and the peak fraction stored. The purified FBPase protein was used as antigen to prepare the antibody. The recombinant Trx-*f* was expressed in *E. coli* and purified as described previously (39).

AMS labeling of the γ subunit of CF_1. The redox state of the γ subunit was determined by the standard procedure reported in (37, 40) using the specific thiol-labeling reagent AMS, which only labels free thiol groups of cysteine residue. For complete oxidation or reduction, CF_1 in 300 mM sucrose and 2 mM Tricine-NaOH (pH 8.0) was incubated for 90 min at 25°C with 50 μM diamide or 20 mM DTT and 19 μM Trx-*f*. After incubation, CF_1 was precipitated by the addition of the ice-cold 10% (v/v) trichloroacetic acid and collected by centrifugation for 10 min at 10,000 × *g* at 4°C. The precipitate was washed twice with the ice-cold acetone and then suspended into 4 mM AMS, 125 mM Tris-HCl (pH 7.5) and 1% (w/v) SDS for thiol modification. The solution was incubated for 1 h at 25°C and the proteins in the solution separated by SDS-PAGE using 12% (w/v) polyacrylamide gel. The γ subunit was visualized using a specific antibody against γ and the redox level was determined from their mobility on the gel as follows. For the calculation of the reduced/oxidized proteins, the sample size loaded on the gel was controlled to avoid saturation of the signal after Western Blotting.

Determination of the reduction/oxidation level of thiol enzymes. Following the separation of the reduced and oxidized proteins by SDS-PAGE, which were modified using AMS, the protein band was visualized by Western blotting using the specific antibody. The visualized band intensity was then captured using the imaging analyzer

LAS-3000mini (Fuji Film, Tokyo, Japan) and quantified by Scion Image software (Scion Co., USA). The redox level of thiol enzyme was determined by the following formula:

Reduction/Total (%) = 100 × *Ired*/(*Ired* + *Iox*), where *Ired* is the integral value of the band intensity of the reduced form protein and *Iox* the integral value of that of the oxidized one. In the case of the γ subunit, the intensity ratio 1:5.4 was included in the equation; Reduction/Total (%) = 100 × 5.4 *Ired*/(5.4 *Ired* + *Iox*),.

Photosynthetic reduction of thiol-enzymes in the intact chloroplasts. Freshly prepared intact chloroplasts (0.2 mg chlorophyll/ml) were incubated for 10 min at 25°C in the dark, and illuminated for 10 min at 25°C with the various light intensities. To avoid the change in temperature of the sample solution by illumination, a 5 cm water filter was placed between the lamp and the sample. After turning off the light, the chloroplasts were incubated in the dark. During this dark/light/dark transition, small aliquots (50 μl) were collected from the chloroplast solution at an appropriate time interval and directly mixed with the thiol group labeling solution containing 4 mM AMS, 125 mM Tris-HCl (pH 7.5) and 1% (w/v) SDS. The mixture was incubated for 60 min at 25°C. After centrifugation for 10 min at 10,000 × *g* at 25°C, supernatant proteins were separated by SDS-PAGE and the redox states of the γ subunit of CF_1 and FBPase were visualized using specific antibodies, and the band intensity quantified as described.

Photosynthetic activation of the ATP hydrolysis activity of the chloroplast ATP synthase in intact chloroplasts. The freshly prepared intact chloroplasts (70 μg chlorophyll/ml) were incubated in the same dark/light/dark conditions as those for the

experiments for evaluation of the redox level of the γ subunit. During incubation, small aliquots (20 μl) were collected with an appropriate time interval and directly added to 180 μl of ATPase assay mixture containing 2 mM ATP, 50 mM Tricine-NaOH (pH 8.0) and 0.5 mM $MgCl_2$ at 37°C. The mixture was incubated for 30 min and terminated by the addition of 2.4% (v/v) perchloric acid. Liberated phosphate was measured by colorimetric method after formation of the Mo-phosphate coordinate (41).

Concentration of ATP in chloroplasts. At the time indicated, 50 μl of chloroplast aliquots were withdrawn and added to 10 μl of 12% (w/v) perchloric acid. 50 μl of the supernatant were neutralized with 125 μl of 2 M Tris-acetate (pH 7.7), and ATP was quantified by a luciferin-luciferase assay using ATP bioluminescence assay kit CLS II (Roche Diagnostics, Basel, Switzerland).

Photoreduction of the thiol enzymes in field-grown spinach leaves. Photosynthetic reduction of thiol enzymes in field grown spinach leaves was determined using specific AMS labeling of thiol groups. To avoid the influence of the artificial light particularly in the dark, the spinach planter was placed on the roof of the isolated building in the campus. At the time indicated in Fig. 7, a spinach leaf specimen was collected from the plants and placed directly into liquid nitrogen to fix the reduced or oxidized situation of the thiol enzymes in the cells. The frozen leaf was directly grinded in the liquid nitrogen and the powder obtained was placed into the thiol group labeling solution containing 4 mM AMS, 125 mM Tris-HCl (pH 7.5), and 10 mM EDTA with 8 M urea to monitor FBPase and without urea to monitor CF_1-γ. The sample was incubated for 30 min at 25°C to complete the labeling of thiol groups with AMS. The hydrophilic protein

fraction was then isolated by addition of an equal volume of chloroform to the sample and the aqueous portion was collected after centrifugation. All proteins in the sample solution were then precipitated by 10% trichloroacetic acid. After washing twice with cold acetone, 125 mM Tris-HCl (pH 7.5) and 1% SDS were added to solubilize precipitates. The redox levels of γ and FBPase were visualized by Western blotting after SDS-PAGE using 12% (w/v) polyacrylamide gel.

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FIGURE LEGENDS

Fig. 1. Dependency of photoreduction of CF_1-γ in the intact chloroplasts on light intensity. (A) Following incubation for 10min in the dark, intact chloroplasts (0.2 mg chlorophyll/ml) were illuminated for 10 min at the indicated light intensities and then incubated for an additional 10 min in the dark. At an appropriate time interval, the sample was mixed directly with the thiol group labeling solution containing AMS, and the redox level of the γ subunit in the intact chloroplasts visualized using the CF1-γ antibody following SDS-PAGE. (B) The redox level of the γ subunit in intact chloroplasts was determined by the method described in 'Materials and Methods' from the band intensities shown in Fig. 1A. (●) 12.5, (○) 25, (▲) 50, (△) 100, (■) 800, (□) 1600 μmol photons·m^{-2}·s^{-1}.

Fig. 2. Effects of uncoupler and electron transport inhibitors on photoreduction of the γ subunit. (**A**) Following incubation for 10 min in the dark, intact chloroplasts (0.2 mg chlorophyll/ml) were illuminated at 800 μmol photons·m^{-2}·s^{-1} for 10 min and then incubated for an additional 5 min in the dark (●). 1 min after illumination, (▲) 20 μM DBMIB, (△) 10 μM DCMU, and (○) 2.5 μM FCCP were added to the chloroplast solution in the light (down arrow), and the redox level of the γ subunit was determined at the time indicated. The results of three independent experiments were averaged. The size of the standard error is shown on each plot. (**B**) Effects of uncoupler and electron transport inhibitors on the ATP hydrolysis activity were examined. The intact chloroplast solution (70 μg chlorophyll/ml) was illuminated at 800 μmol photons·m^{-2}·s^{-1}, and the inhibitors were added to the chloroplasts as for (A). The ATP hydrolysis

activities were determined by the method described in 'Materials and Methods'. The results of three independent experiments were averaged and the size of the standard error is shown on each plot.

Fig. 3. Effects of uncoupler and electron transport inhibitors on photoreduction of FBPase. Following incubation for 10 min in the dark, intact chloroplasts (0.2 mg chlorophyll/ml) were illuminated at 800 μmol photons·m^{-2}·s^{-1} for 10 min and then incubated for an additional 5 min in the dark (●). 1 min after illumination, (▲) 20 μM DBMIB, (△) 10 μM DCMU, and (○) 2.5 μM FCCP were added to the chloroplast solution in the light (down arrow), and the redox level of FBPase was determined at the time indicated. The results of three independent experiments were averaged. The size of the standard error is shown on each plot.

Fig. 4. ATP level in the intact chloroplasts under different light intensities. (A) Intact chloroplasts (0.2 mg-chl/ml) were incubated for 10min in the dark, illuminated at 25 μmol photons·m^{-2}·s^{-1} (▲), 100 μmol photons·m^{-2}·s^{-1} (○), and 800 μmol photons·m^{-2}·s^{-1} (●) for 10 min, and incubated for an additional 5 min in the dark. At the indicated time, a portion of the chloroplast samples were mixed directly with TCA, and the amounts of ATP determined by the method described in 'Materials and Methods'. The results of three independent experiments were averaged and the size of the standard error is shown on each plot. (B) Following incubation for 10 min in the dark, intact chloroplasts were illuminated at 800 μmol photons·m^{-2}·s^{-1} for 10 min and then incubated for an additional 5 min in the dark (closed circle). 1 min after illumination, 2.5 μM FCCP was added to the chloroplast solution in the light (down arrow, open circle).

Fig. 5. Photoreduction in field-grown spinach leaves. Field grown spinach leaves in the planter were collected at the indicated time and the redox states of the thiol-enzymes were visualized by the method as described. The redox level of the γ subunit (○), FBPase (●), and change of the light intensity (▲) were indicated. The results of two independent measurements are shown; (**A**) the sampling was carried out on 30, Oct. 2008. The weather was occasionally cloudy all day. (**B**) the sampling were carried out on 11, Nov. 2008. The weather was cloudy all day.

A

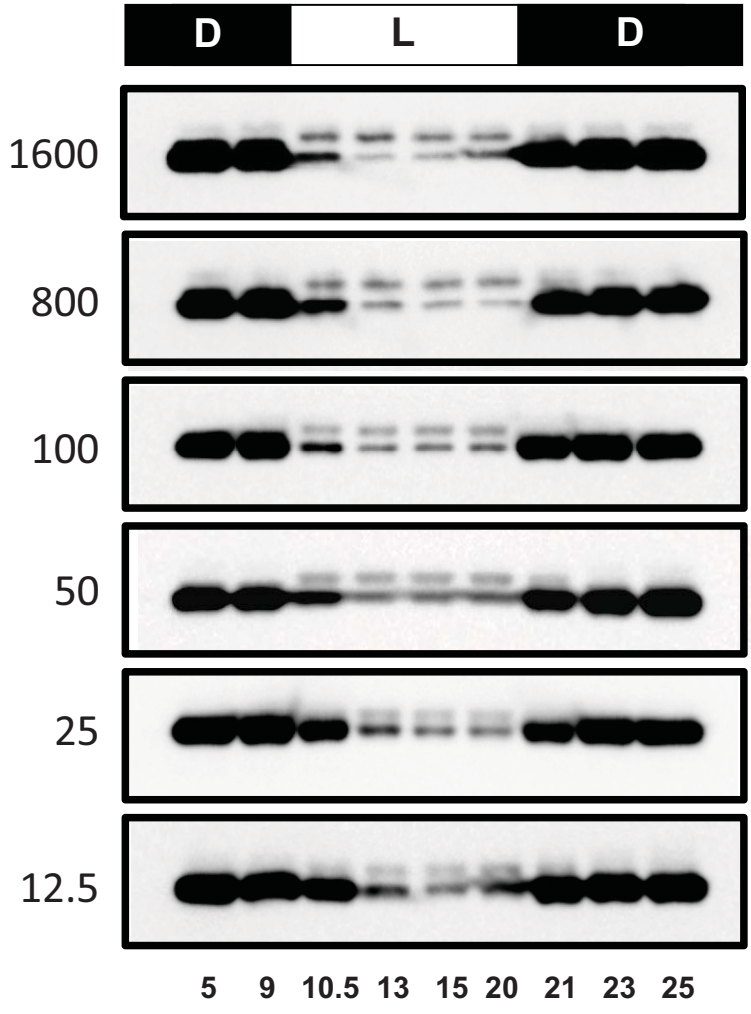

Fig. 1

B

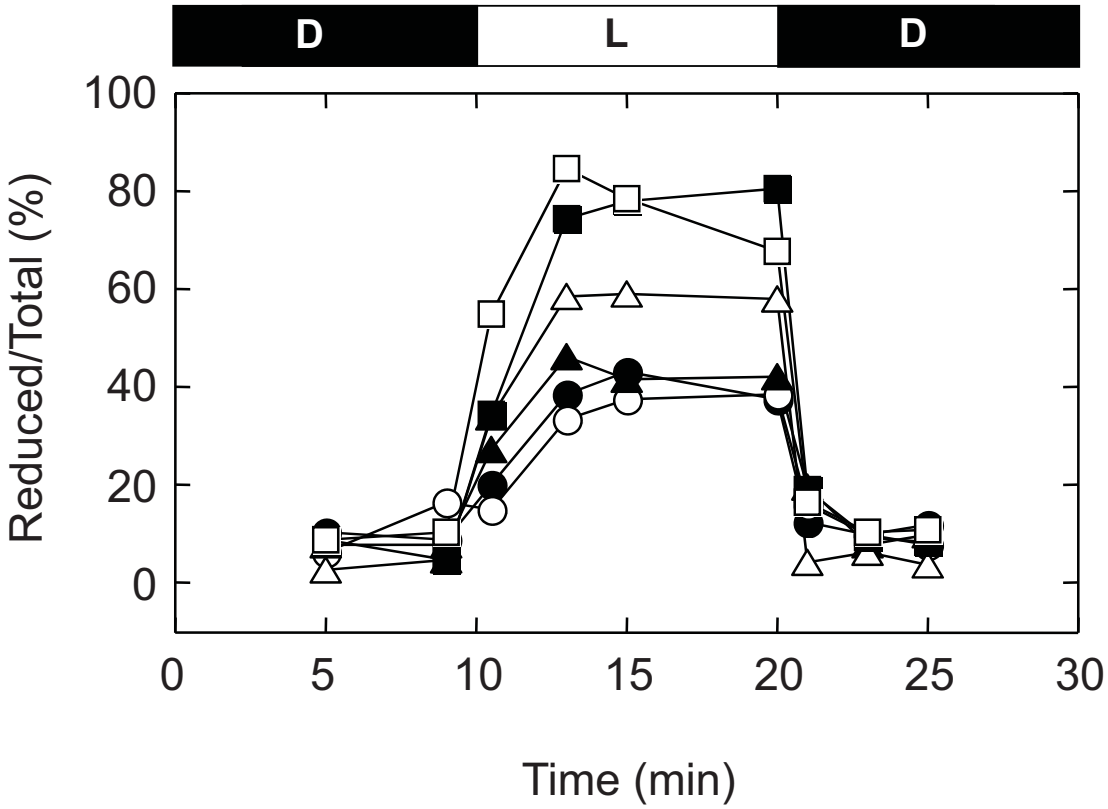

Fig. 1

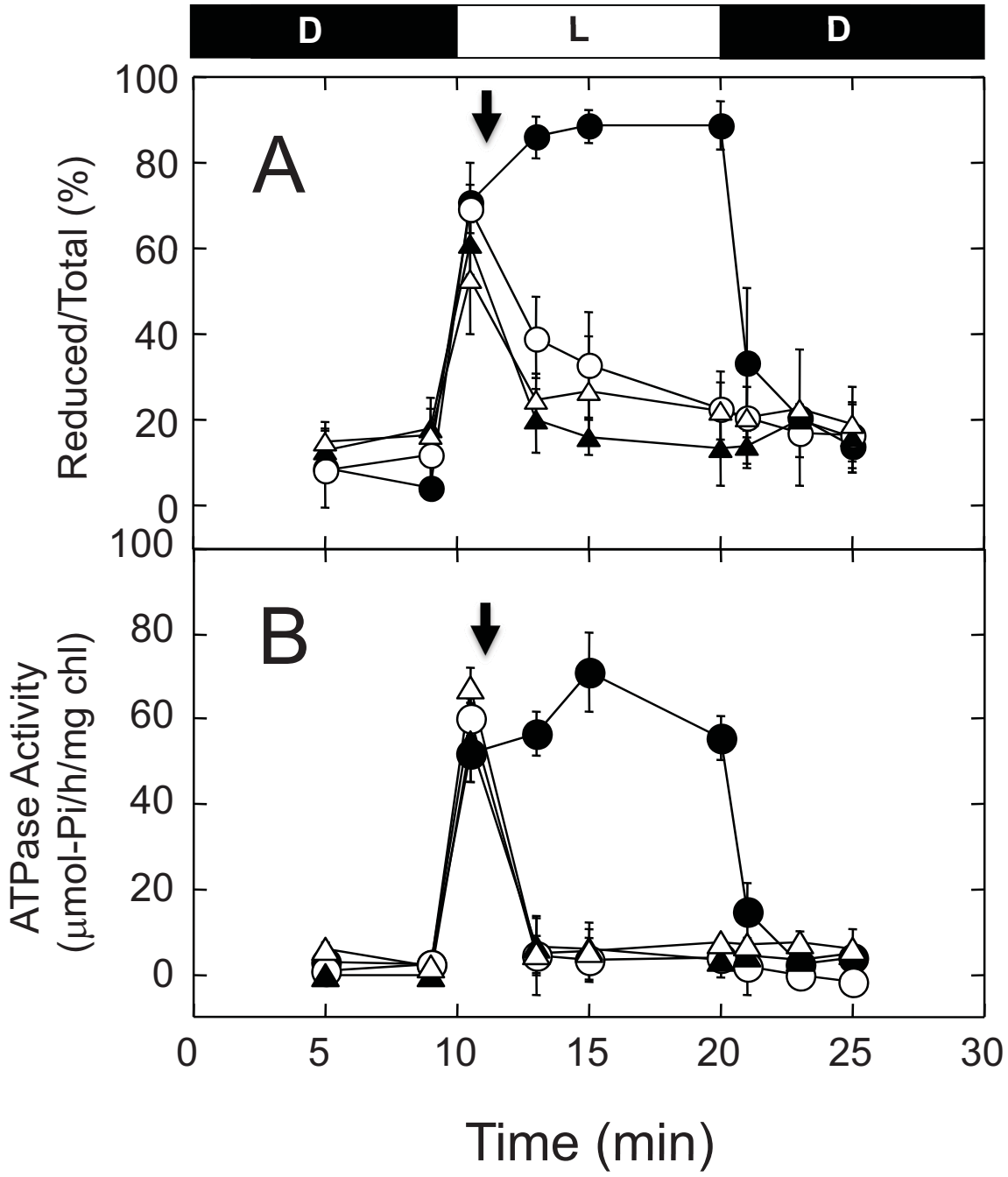
D
L
D
A
B
Reduced/Total (%)
ATPase Activity
(μmol-Pi/h/mg chl)
0
20
40
60
80
100
0
5
10
15
20
25
30
Time (min)

Fig. 2

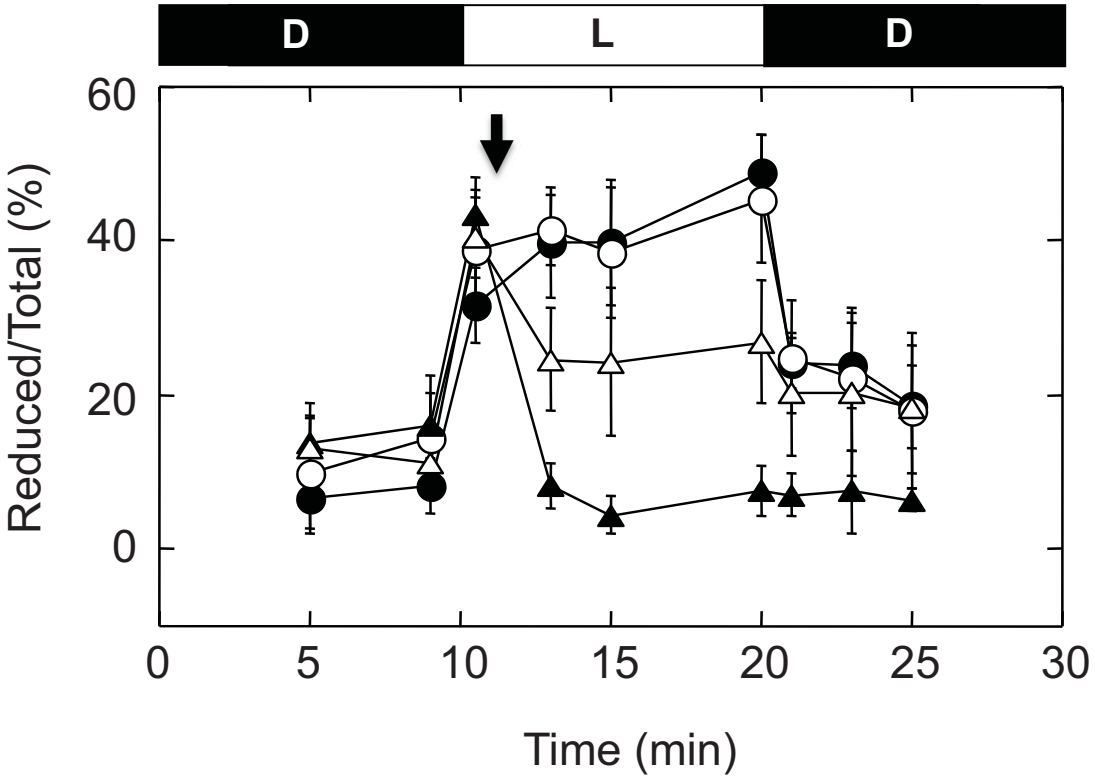

Fig. 3

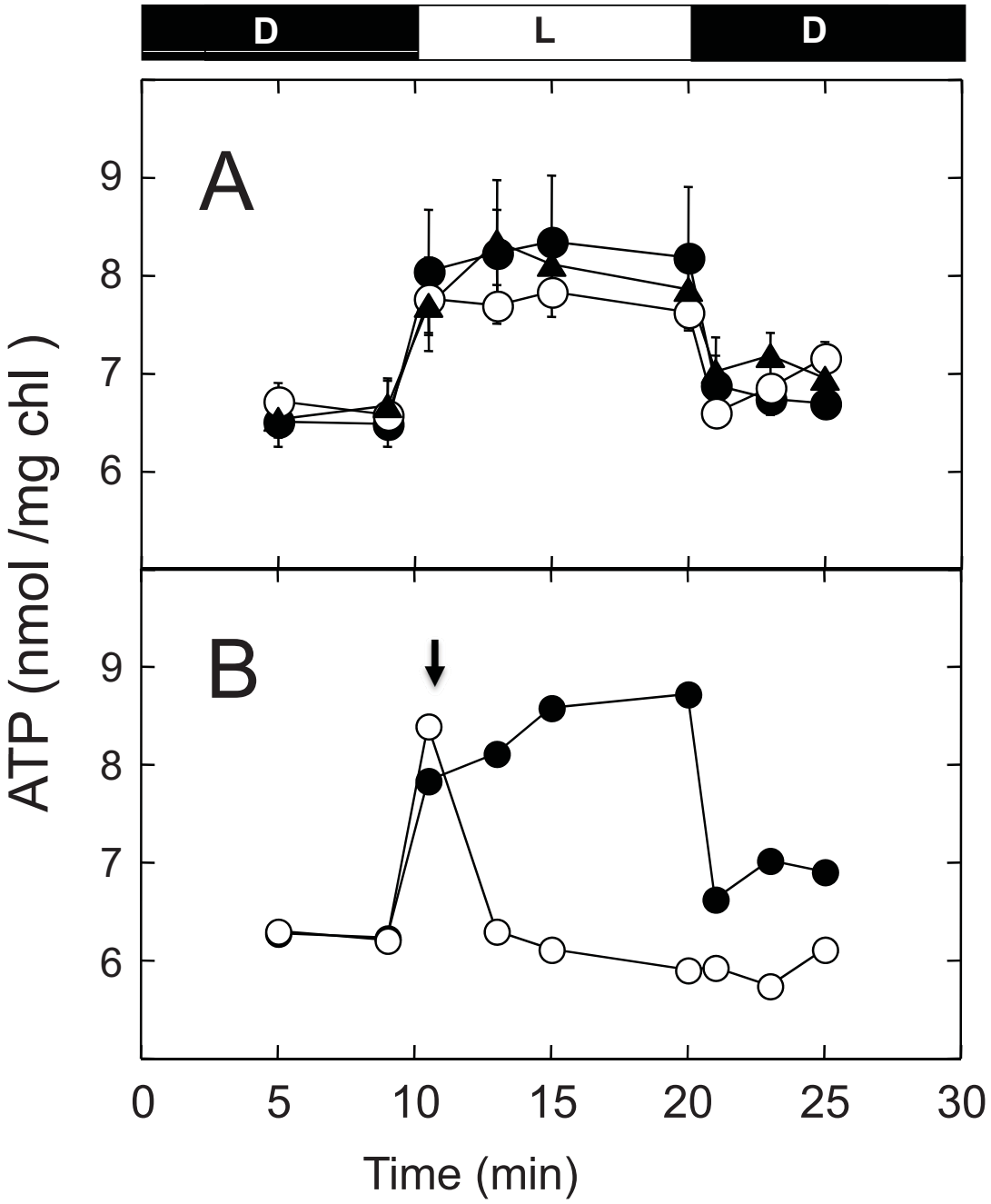

Fig. 4

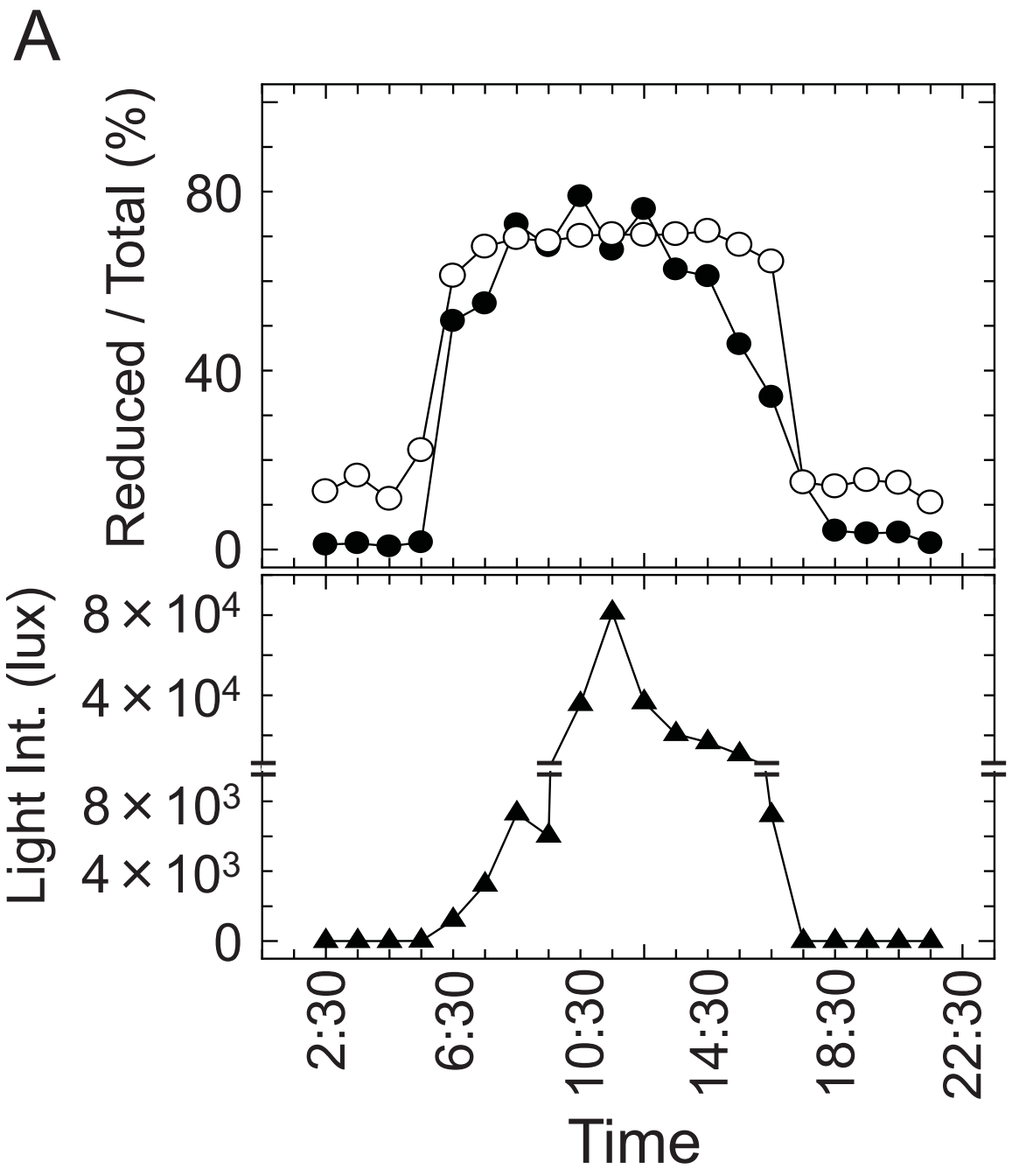
A
Reduced / Total (%)
80
40
0
Light Int. (lux)
8 × 10^4
4 × 10^4
8 × 10^3
4 × 10^3
0
2:30
6:30
10:30
14:30
18:30
22:30
Time

Fig. 5

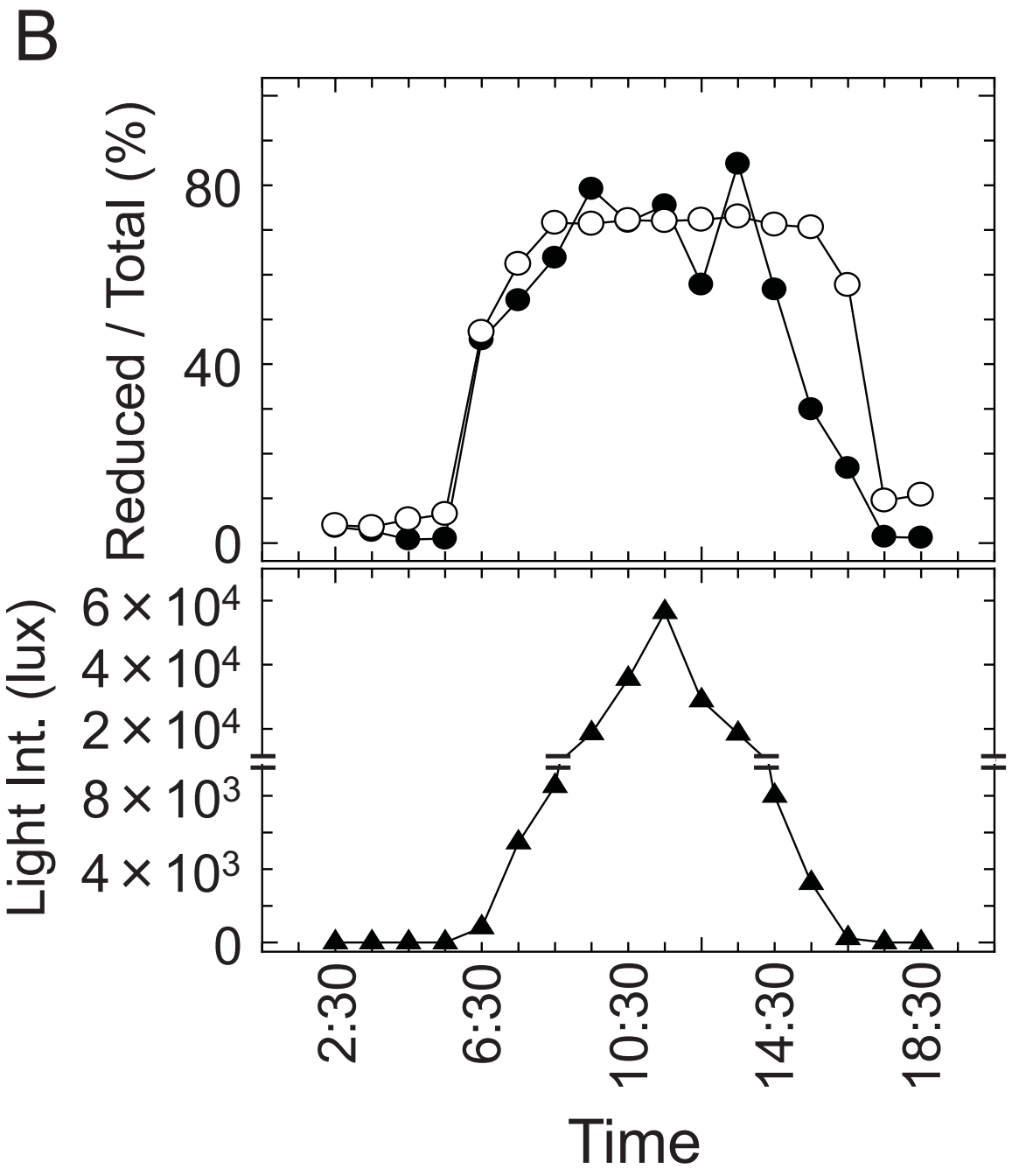
B
Reduced / Total (%)
80
40
0
Light Int. (lux)
6 × 10^4
4 × 10^4
2 × 10^4
8 × 10^3
4 × 10^3
0
2:30
6:30
10:30
14:30
18:30
Time

Fig. 5