Distinct Redox Behaviors of Chloroplast Thiol Enzymes and their Relationships with Photosynthetic Electron Transport in Arabidopsis thaliana

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The thiol/disulfide redox network mediated by the thioredoxin (Trx) system in chloroplasts ensures light-responsive control of diverse crucial functions. Despite the suggested importance of this system, the working dynamics against changing light environments remains largely unknown. Thus, we directly assessed the in vivo redox behavior of chloroplast Trx-targeted thiol enzymes in Arabidopsis thaliana. In a time-course analysis throughout a day period that was artificially mimicked to natural light conditions, thiol enzymes showed a light-dependent shift in redox state, but the patterns were distinct among thiol enzymes. Notably, enzymes showed a light-dependent shift in redox state, but was artificially mimicked to natural light conditions, thiol enzymes showed a light-dependent shift in redox state, but the patterns were distinct among thiol enzymes. Notably, the ATP synthase CF1-subunit was rapidly reduced even under low-light conditions, whereas the stromal thiol enzymes fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, and NADP-malate dehydrogenase were gradually reduced/re-oxidized along with the increase/decrease in light intensity. Photo-reduction of thiol enzymes was suppressed by the impairment of photosynthetic linear electron transport using DCMU and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, but sensitivity to the impairment was uneven between CF1-subunit and other stromal thiol enzymes. These different dependencies of photo-reduction on electron transport, rather than the redox state of Trx and the circadian clock, could readily explain the distinct diurnal redox behaviors of thiol enzymes. In addition, our results indicate that the cyclic electron transport around PSI is also involved in redox regulation of some thiol enzymes. Based on these findings, we propose an in vivo working model of the redox regulation system in chloroplasts.

Keywords: Arabidopsis thaliana • Chloroplast • Photosynthetic electron transport • Redox regulation • Thiol enzymes • Thioredoxin (Trx).

Abbreviations: AL, actinic light; AMS, 4-acetoamido-4′-malimidylstilbene-2,2′-disulfonate; AntA, antimycin A; CET, cyclic electron transport around PSI; CF1, catalytic moiety of ATP synthase; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; FBPase, fructose 1,6-bisphosphatase; Fd, ferredoxin; FTR, ferredoxin-thioredoxin reductase; H2O2, hydrogen peroxide; LET, linear electron transport; MDH, NADP-malate dehydrogenase; NDH, NADPH dehydrogenase; NTA, nitrilotriacetic acid; PGR5, proton gradient regulation 5; ROS, reactive oxygen species; SBPase, sedoheptulose 1,7-bisphosphatase; SP, saturating pulse; Trx, thioredoxin.

Introduction

Proteins located in chloroplasts need to be flexibly and suitably regulated under fluctuating light environments to ensure efficient chloroplast functions. The electron transport chain in the thylakoid membrane converts light to ATP and NADPH during photosynthesis, which are mainly used for the Calvin cycle reaction in the stroma. Photosynthetic electron transport also initiates the thiol/disulfide redox cascade by branching electrons at the site of ferredoxin (Fd). Reducing equivalents are then transferred to specific proteins containing redox-active Cys residues (referred to as thiol enzymes), allowing modulation of their enzymatic activities. Therefore, the thiol/disulfide redox cascade confers several target systems in chloroplasts on the regulatory way coordinated by light. The key machinery mediating the reducing equivalent transfer from the electron transport chain to thiol enzymes is the thioredoxin (Trx) system composed of Fd-Trx reductase (FTR) and Trx.

Trx is a small ubiquitous protein with an active site containing one pair of Cys residues that undergoes reversible reduction and oxidation. Trx was first identified in Escherichia coli in 1964 as a ribonucleotide reductase cofactor (Laurent et al. 1964). In the 1970s, members involved in the Trx system, such as Trx and FTR, were identified in chloroplasts (Buchanan and Wolosiuk 1976, Schürmann et al. 1976, Wolosiuk and Buchanan 1977). It was further demonstrated that four Calvin cycle...
enzymes, namely fructose 1,6-bisphosphatase (FBPase), sedoheptulose 1,7-bisphosphatase (SBPase), NADP-glyceraldehyde 3-phosphate dehydrogenase, and phosphoribulokinase are activated when the Trx system is reconstituted in vitro (for a recent review, see Michelet et al. 2013). These reports led to the establishment of the current basic concept regarding the chloroplast Trx system; Trx receives reducing equivalents from the light-driven photosynthetic electron transport chain through FTR and subsequently reduces specific disulfide bridges on thiol enzymes. Further study using intact chloroplasts indicated that this machinery is physiologically functional (Crawford et al. 1989).

Proteins subjected to Trx-dependent thiol modulation are not restricted to Calvin cycle enzymes. Early studies showed that Trx participates in the activation of ATP synthase (McKinney et al. 1979). Thereafter, two adjacent Cys residues localized in the central axis of the CF₁-γ subunit were revealed to have a critical role in redox regulation of ATP synthase (Nalin and McCarty 1984, Miki et al. 1988). Proteins mediating other metabolic pathways such as NADP-malate dehydrogenase (MDH) involved in the malate valve have been also known to be redox-regulated through the Trx system (Scheibe 1981) but understanding of the Trx-targeted thiol enzyme remained limited until 2000. Information on the Trx target protein was drastically broadened in 2001 by the development of methodology for capturing Trx-interacting proteins (Motohashi et al. 2001, Yano et al. 2001). Accompanied by advanced proteomic techniques, these strategies have been used to explore Trx target candidates not only in chloroplasts but also in other cellular compartments including cytosol (Yamazaki et al. 2004) and mitochondria (Balmer et al. 2004, Yoshida et al. 2013). This has lead to the discovery of Trx-linked proteins associated with a broad spectrum of cellular processes (see Montrichard et al. 2009 and Lindahl et al. 2011 for reviews).

The plant Trx system is characterized by divergent Trx subtypes. At least 20 Trx genes are divided into seven classes (f, m-, h-, o-, x-, y, and z-type) in Arabidopsis thaliana. Among them, as many as five classes (f, m-, x-, y, and z-type) are targeted to chloroplasts. Trx-f and Trx-m were initially identified as the light-dependent activators that preferentially reduce FBPase and MDH, respectively (Schürmann et al. 1981). Trx-x and y were newly discovered through the increased availability of plant genome information and efficiently donate reducing equivalents to the anti-oxidant defense system (Collin et al. 2003, Collin et al. 2004). Trx-z was recently identified as a novel Trx required for chloroplast development by regulating transcription in chloroplast genes (Arsova et al. 2010). Besides these Trx subtypes, NADPH-Trx reductase C is also localized to chloroplasts (Serrato et al. 2004) and mediates redox regulation of several processes, including anti-oxidant defense (Pérez-Ruiz et al. 2006), starch synthesis (Michalska et al. 2009), and tetrapyrrole metabolism (Richter et al. 2013). These findings suggest that chloroplasts host a complex redox network, although the functional diversity is yet to be fully clarified (see König et al. 2012 and Serrato et al. 2013 for reviews).

As summarized above, many previous efforts with a long history have substantially contributed to the knowledge of the Trx-mediated redox pathway and its possible target proteins in chloroplasts. Thanks to the direct determination of enzyme structure, molecular mechanisms of the redox regulation system are becoming increasingly apparent (Michelet et al. 2013). However, because the current understanding has been achieved by biochemical in vitro assays or proteomics-based analyses, it is nearly completely unclear how the chloroplast Trx system works in vivo. Using a method to visualize the in vivo redox state, we recently demonstrated that the ATP synthase CF₁-γ subunit in spinach leaves shows a drastic shift in redox state throughout the day (Konno et al. 2012). Here, we performed a comparative study of the in vivo redox behaviors of several thiol enzymes in A. thaliana to obtain more comprehensive insight into the chloroplast redox regulation system in living cells. We also addressed these regulatory mechanisms with a focus on the association with photosynthetic electron transport. We provide evidence that redox behaviors to changing light environments are not uniform among thiol enzymes, which results from different electron transport efficiency relationships.

**Results**

**Chloroplast thiol enzymes show distinct diurnal redox behaviors**

We first assessed the diurnal dynamics of in vivo thiol modulation. For this purpose, a growth chamber was programmed to mimic light conditions in the field (Fig. 1A). Light intensity was gradually and almost linearly elevated under programmed conditions and reached approximately 350 μmol photons m⁻² s⁻¹ at 5 h after the onset of illumination. Light intensity was then gradually attenuated and completely turned off at 11 h after the onset of illumination. During this period, Arabidopsis wild-type plants were placed in the chamber and sequentially sampled, followed by determining the reduction level of each thiol enzyme and Trx (see Materials and Methods).

Our method for visualizing the in vivo redox state is based on the difference in protein mobility on non-reducing SDS-PAGE between the reduced [regulatory Cys modified with 4-acetoamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS)] and oxidized (unmodified) forms (Motohashi et al. 2001). As shown in Fig. 1B, we observed a clear band shift in several thiol enzymes depending on the change in light environment. Interestingly, the observed redox behaviors were not uniform among thiol enzymes (Fig. 1C). The ATP synthase CF₁-γ subunit showed a rapid shift from the oxidized to the reduced form at the very beginning after turning on the light when light intensity was still weak. CF₁-γ was maintained in almost a fully reduced state until the light was completely turned off. In contrast, FBPase and SBPase were reduced slowly concomitant with the increase in light intensity, followed by gradual
Redox dynamics under decreasing light intensity. Although the induction pattern of photo-reduction was similar between FBPase and SBPase, the saturating reduction level was different; FBPase reached >80% of full reduction, whereas SBPase reached only approximately 60%. MDH did not show a clear band shift between oxidized and reduced forms (Fig. 1D). This was possibly due to the fact that more than one pair of Cys residues are involved in the redox regulation of MDH (Miginiac-Maslow et al. 2000). Nevertheless, it was apparent that MDH was converted gradually from an oxidized to a reduced form by increasing light intensity, and vice versa by decreasing light intensity.

We also examined the redox state of two Trx isoforms, Trx-m2 and Trx-f2 (Fig. 1B, C). Three discriminative bands were observed for Trx-f2, which may have been related to the cross-reaction with Trx-f1 or the glutathionylation of the additional Cys conserved in Trx-f (Michelet et al. 2005). As the most upper band emerged after illumination, the reduction level of Trx-f2 was quantified on the assumption that the upper band corresponded to the reduced form. Partial conversion to the reduced form was observed for both in Trx-m2 and Trx-f2 upon illumination. However, these responses were not so drastic toward the change in light intensity and the reduction levels were kept almost stable (<50% of full reduction) throughout a day period.

**The diurnal redox behaviors of chloroplast thiol enzymes are linked to light intensity, not the circadian clock.**

We next examined whether circadian clock-dependent regulation is involved in diurnal dynamics of thiol modulation. Following a gradual increase, light intensity was maintained at a saturating level until 11 h after the onset of illumination (Fig. 2A). Under such prolonged light conditions, the reduction levels of all thiol enzymes and Trxs examined here were kept at saturating states (Fig. 2B, C), indicating that diurnal redox behaviors of thiol enzymes are absolutely linked to light intensity, not the circadian clock.
Photosynthetic linear electron transport efficiency differentially affects the photo-reduction pattern of each thiol enzyme

The Trx system is associated with light through the photosynthetic electron transport chain in the thylakoid membrane. Photosynthetic electron transport consists of linear electron transport (LET) from water to NADP⁺ and cyclic electron transport around PSI (CET). Although the contribution of CET in photosynthesis is still a matter of intense debate, several lines of evidence show that this pathway is necessary for driving thermal dissipation of excess light energy and avoiding over-reduction of PSI (for a review, see Shikanai 2007). In order to gain insights into the relationship between redox dynamics and the efficiency of each photosynthetic electron transport, we examined the photo-reduction patterns of thiol enzymes under conditions where LET and CET are individually impaired.

Leaves were treated with DCMU (inhibitor of electron transfer between Q₂ and Q₀ in PSI) or 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB; inhibitor of electron transfer between plastoquinone and the cytochrome b₅/f complex) to restrict LET. The inhibitory effects were then validated by monitoring Chl fluorescence (Fig. 3A). When leaves were treated with 10 µM DCMU or DBMIB, the basal level of Chl fluorescence under actinic light (AL) was significantly elevated. However, a saturating pulse (SP) caused a transient increase in Chl fluorescence, indicating that LET was still partially functional. In contrast, when each inhibitor was used at 100 µM, the basal level of Chl fluorescence reached nearly a maximum level and SP no longer excited Chl fluorescence. It was thus confirmed that LET was completely impaired in the presence of 100 µM of each inhibitor.

Inhibitor-treated leaves were illuminated at two different intensities (low light: 30 µmol photons m⁻² s⁻¹; high light: 800 µmol photons m⁻² s⁻¹), followed by visualization of the redox state of the thiol enzymes (Fig. 3B). CF₁-γ in the control treatment was fully reduced under both light regimes, which coincided with the data shown in Fig. 1. In the presence of 10 µM inhibitor in which LET was partly restricted, about half of the CF₁-γ was reduced upon illumination. Interestingly, while CF₁-γ was partially photo-reduced in the presence of 100 µM DCMU, it was completely kept in the oxidized form in the presence of 100 µM DBMIB. Given that DBMIB inhibits both LET and CET, this result suggests that CET is also involved in the redox regulation of CF₁-γ (see below). In contrast to CF₁-γ, photo-reduction of other thiol enzymes, FBPase, SBPase, and MDH, was completely suppressed by DCMU or DBMIB even at...
Redox dynamics of chloroplast thiol enzymes

Effects of linear electron transport (LET) impairment on photo-reduction of several thiol enzymes and thioredoxins (Trxs). (A) Traces of Chl fluorescence in control and LET-impaired leaves. Chl fluorescence is represented as a relative value to the maximum level (Fig. 1). Light intensity during the treatment is indicated above the image (30 and 800 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)). Experiments were repeated three times using different sample preparations and the representative results are shown.

Cyclic electron transport around PSI is involved in the redox regulation of some thiol enzymes

We next addressed whether CET has an impact on thiol modulation. For this purpose, CET inhibitor antimycin A (AntA) was applied to leaves, and the effects on photo-reduction of thiol enzymes were examined (Fig. 4). The reduction levels of CF1-\(\gamma\) and FBPass were slightly higher in AntA-treated leaves than those in the control under low-light conditions. Conversely, the reduction level of FBPass was lowered when leaves were treated with AntA under high-light conditions. AntA did not affect photo-reduction of SBPass under either light condition.

AntA is also a typical inhibitor of electron transfer in the mitochondrial respiratory chain, and mitochondrial metabolism supports photosynthesis through organelle crosstalk in illuminated leaves (Noguchi and Yoshida 2008). Thus, we could not rule out the possibility that the results shown in Fig. 4 were caused by impairment of the respiratory chain rather than CET. Therefore, the involvement of CET in thiol modulation was also validated using the proton gradient regulation5 (pgrs) mutant, which fails to perform AntA-sensitive CET (Munekage et al. 2002). The photo-reduction patterns of the thiol enzymes were compared between wild-type and pgrs at several light intensities (Fig. 5A, B). Under low-light conditions, the reduction levels of CF1-\(\gamma\) and FBPass were slightly higher in pgrs than in the wild type. FBPass was almost fully reduced in the wild-type under high-light conditions, whereas 20–30% FBPass was present in the oxidized form in pgrs. Genetic impairment of CET did not significantly affect photo-reduction of SBPass as was the case with AntA treatment.

CET is also mediated by another pathway catalyzed by the AntA-insensitive NADPH dehydrogenase (NDH) complex. This pathway is not functional in the chlororespiratory reduction2 (crr2) mutant due to the lack of proper NDH complex formation (Hashimoto et al. 2003). By treating mutants with AntA, we assessed the difference in the contribution of PGRS- and NDH-dependent CET to thiol modulation under high-light conditions (1,100 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), Fig. 5C). Photo-reduction of CF1-\(\gamma\) was not affected by any impairments in CET. FBPass was partly present in the oxidized form in pgrs, which was no longer affected by AntA due to the intrinsic lack of an AntA-sensitive pathway. FBPass was fully photo-reduced in crr2 in the absence of AntA, but was partly oxidized by AntA treatment. This
AntA was used at 10 𝜇M or 100 𝜇M to inhibit CET. Experiments were performed as described in the legend of Fig. 3. (B) Effects of AntA treatment on the reduction level of the ATP synthase CF1-γ subunit, FBPase, and SBPase. The reduction level was quantified as the ratio of the reduced form to the total. Immunoblotting analysis was repeated six times using three different sample preparations (two analyses per sample preparation) and the mean ± SD is shown. Different letters denote significant differences among treatments (P < 0.05, Tukey-Kramer multiple comparison test).

Discussion

Chloroplast redox regulation through the Trx system has been generally acknowledged as an important system for controlling diverse functions in response to light. However, while our understanding of redox regulation has grown widely at the biochemical and structural levels, a quite fundamental but physiologically important question of how this system responds to light has remained unanswered. Thus, we assessed the redox dynamics of several thiol enzymes in illuminated leaves and their regulatory mechanisms.

Distinct diurnal redox behaviors between ATP synthase CF1-γ subunit and stromal thiol enzymes are primarily attained by different dependencies on LET

As shown in Fig. 1, we observed distinct diurnal redox behaviors of thiol enzymes. The most remarkable feature was found in the ATP synthase CF1-γ subunit, which was almost fully reduced even under low-light conditions. Using the electrochromic shift assay, Kramer et al. (1990) reported that ATP synthase is fully activated even under quite low-light conditions where the CO2 fixation rate is still not saturated. As ATP synthase is regulated by a multi-step mechanism (for a review, see Hisabori et al. 2013), it was unclear only from their study whether this activation was linked to thiol modulation of CF1-γ. Our present data directly visualizing the in vivo redox state strongly indicate that rapid photo-reduction of CF1-γ enables ATP synthase to be activated even under light-limited conditions. In contrast, the stromal enzymes FBPase, SBPase, and MDH were gradually photo-reduced/re-oxidized concomitant with the increase/decrease in the light intensity. What factor controls these distinct responses? In general, LET in the thylakoid membrane is accelerated in parallel with the increase in light intensity and reaches the saturating rate under high-light conditions (e.g. Yoshida et al. 2011), although we could not monitor the diurnal pattern of LET efficiency due to the technical limit of the measurement. In experiments using LET inhibitors, we found that CF1-γ was photo-reduced even when LET was partially restricted, whereas photo-reduction of other stromal thiol enzymes was completely abolished under the same conditions (Fig. 3). These results indicate that photo-reduction of CF1-γ can be sensitively triggered in response to subtle induction of LET, while others cannot be photo-reduced until LET is substantially activated (Fig. 6).

The transcript levels of Trx genes fluctuate through circadian regulation (Barajas-López et al. 2011) or in response to the accumulation of photosynthetic products (Barajas-López et al. 2012). Furthermore, recent reverse-genetic studies have
demonstrated that manipulating Trx accumulation level leads to a change in the redox state of some thiol enzymes. For example, disruption of Trx-f1 gene results in impaired photo-reduction of ADP-glucose pyrophosphorylase in Arabidopsis (Thormählen et al. 2013). Therefore, the amount or redox states of Trxs are also candidates for determining diurnal redox behavior of thiol enzymes. However, a clear change in protein abundance of Trx-m2 and Trx-f2 was not observed throughout a day period (Fig. 1). More notably, the redox states of Trxs did not shift drastically regardless of light intensity and were maintained at <50% of full reduction (Fig. 1). Given that the impairment of LET abolished the photo-reduction of Trxs (Fig. 3), Trx must be located on a route necessary for transferring reducing equivalents from photosynthetic electron transport to thiol enzymes. However, the results shown in Fig. 1 suggest that the amount and redox state of Trx are not rate limiting for regulating diurnal redox behaviors of thiol enzymes, at least under non-stressful conditions. We also addressed whether circadian clock-dependent regulation is directly involved in thiol modulation (Fig. 2). The results clearly demonstrated that diurnal redox behaviors of thiol enzymes are not regulated by the circadian clock, but are strictly linked to light intensity. Taken together, these results allowed us to conclude that different dependencies on LET primarily confer CF1-γ and stromal thiol enzymes to distinct diurnal redox behaviors (Fig. 6).

An intriguing question is then raised about the mechanism of different dependencies on LET between CF1-γ and stromal thiol enzymes. It appears difficult to explain this mechanism only by a difference in midpoint redox potential ($E_m$), because the reported $E_m$ values of thiol enzymes including CF1-γ are not much different from that of Trx (Kramer and Crofts 1989, Hirasawa et al. 1999, Hutchison et al. 2000). The most plausible hypothesis is that it results from different localization in chloroplasts. When LET is activated, reducing equivalents may be easily transferred to CF1-γ (peripherally residing in the thylakoid membrane) more than other thiol enzymes (drifting in the stroma). In support of this idea, the membrane-bound...
How is CET involved in redox regulation of chloroplast thiol enzymes?

Another intriguing finding in this study is that CET was involved in redox regulation of some thiol enzymes (Figs. 4, 5). Courteille et al. (2013) demonstrated that Trx-m4 exerts negative control on CET. Our results showed that both chemical and genetic impairments of PGR5-dependent CET slightly but significantly elevated the reduction level of CF1-γ and FBPase under low-light conditions (Figs. 4, 5). These results indicate that the Trx system competes with CET for electrons transferred to Fd under light-limited conditions (Fig. 6). This possibility is supported by our previous study showing that the MDH activation state is higher in the pgr5 mutant than that in wild-type plants under low-light conditions (Yoshida et al. 2007). In contrast, impairment of PGR5-dependent CET lowered the reduction level of FBPase under high-light conditions (Figs. 4, 5). As mentioned above, ROS-mediated oxidation of SBPase may occur even in the wild-type under high-light conditions. It is possible that ROS generation is further elevated by blocking CET resulting in PSI over-reduction, which promotes re-oxidation of FBPase, in addition to SBPase (Fig. 6). In support of this idea, it was recently shown by estimating the protein carbonylation level that oxidation power is liable to be exerted in the pgr5 mutant more than in the wild-type (Suorsa et al. 2012). Therefore, CET may be indirectly involved in thiol modulation by suppressing over-reduction of PSI and thereby controlling ROS generation under high-light conditions. These scenarios should be dissected in more detail, but there is little doubt that CET has a significant role in fine tuning of the redox regulation system, and that its mode varies depending on light intensity. It should be noted that the efficiency of CET itself may be under redox regulation through the Trx system (Hertle et al. 2013).

Future perspectives

In this study, we visualized the in vivo diurnal redox dynamics of several thiol enzymes in chloroplasts. Notably, redox behaviors were distinct among thiol enzymes, which was closely associated with electron transport efficiency. These findings provide insight into how chloroplasts adjust their own functions to changing light environments. In order to understand chloroplast redox dynamics more deeply, it is important to clarify other redox-related parameters such as fluctuation of...
NADPH/NADP⁺ ratio and the engagement of anti-oxidant defense systems. Besides our present study, the in vivo redox regulation system, including the distribution of Trx with respect to thiol enzymes (Anderson et al. 2008) and the specific role of each Trx subtype (Chi et al. 2008, Laugier et al. 2013, Thorrnählen et al. 2013) has been recently addressed, but is only starting to emerge. Further investigation of these subjects will promote our understanding of the redox network ensuring light-responsive thiol modulation in chloroplasts.

Materials and Methods

Plant material

*A. thaliana* wild-type plants (Col-0(1)), the pgr5 mutant, and the *err2* mutant (provided courtesy of Prof. T. Shikanai) were grown in soil in a controlled growth chamber (80–90 μmol photons m⁻² s⁻¹, 22 °C, 16 h day/8 h night) for 4 weeks. For the analysis shown in Figs 1 and 2, plants were transferred to a programmed chamber at the end of night period (i.e. just before the onset of program).

Preparation of antibodies

The spinach CF₁-γ antibody used in our previous study showed low affinity to the regulatory Cys-modified reduced form compared with the unmodified oxidized form, possibly because this antibody was raised against the partial polypeptide including the regulatory Cys-containing region (Konno et al. 2012). Thus, a new antibody was generated to improve the accuracy of estimating the CF₁-γ redox state. Recombinant *Arabidopsis* CF₁-γ (His-tagged at the C terminus) was prepared as follows. The ATPC1 (At*6g04640*) gene fragment encoding the mature protein region (Ala⁵¹–Val¹⁷⁷) was cloned into the pET23a expression vector (Novagen, Gibbstown, NJ, USA). The plasmid was transformed to *E. coli* BL21 (DE3), and the CF₁-γ protein was expressed with 0.5 mM IPTG. *E. coli* cells were disrupted with a French pressure cell (5501-M, Ohtake Works, Tokyo, Japan). CF₁-γ, mainly expressed in the inclusion body, was solubilized with 8 M urea in 50 mM phosphate buffer (pH 8.0). After centrifugation at 20,000 × g for 5 min, the resulting supernatant was loaded onto a Ni-nitrilotriacetic acid (NTA) affinity chromatography column (Tosoh, Tokyo, Japan) and QAE-Toyopearl 550C column (Tosoh), and hydrophobic interaction chromatography [using Butyl-Toyopearl 650M (Tosoh)] as described in Yoshiida et al. (2013).

Protein purification was conducted at 4°C. The FBPase antibody was prepared in a previous study (Konno et al. 2012). The SBPase antibody was provided courtesy by Dr. M. Tamoi (Kinki University).

Visualization of the in vivo redox state of thiol enzymes

The in vivo reduction level of chloroplast thiol enzymes was determined according to Konno et al. (2012) with modifications. Plants were placed directly in liquid nitrogen and ground using a mortar and pestle. Proteins were extracted in the SDS sample buffer [2% (w/v) SDS, 62.5 mM Tris·HCl (pH 6.8), 7.5% (v/v) glycerol and 0.01% (w/v) bromophenol blue] containing the protease inhibitor cocktail Complete (Roche, Manheim, Germany) and the specific thiol-labeling reagent AMS (Invitrogen, Carlsbad, CA, USA). The samples were incubated for 60 min at room temperature to complete the labeling of thiol groups with AMS. Non-reducing SDS-PAGE and immunoblotting were performed using a standard method as described in (Yoshida et al. 2007). Protein concentration was determined with a BCA protein assay (Pierce, Rockford, IL, USA).

Inhibitor treatments

Inhibitor treatments to impair photosynthetic electron transport were performed according to Yoshida and Noguchi (2009) with slight modifications. Leaves were excised from plants and vacuum-infiltrated for 5 min with several inhibitor solutions of DCMU, DBMIB, and AntA. As a control, leaves were treated with 1% (v/v) ethanol (used as a solvent for the inhibitors). After vacuum infiltration, leaves submerged in the inhibitor solutions were placed under the indicated light conditions for 30 min. The temperature during treatments was set to 25°C. The inhibitory effects of DCMU and DBMIB on photosynthetic electron transport were confirmed by Chl fluorescence measurements. Chl fluorescence was monitored using mini-PAM (Walz, Effeltrich, Germany).

Statistical analysis

Statistical analyses were conducted with Student’s t-test using Microsoft Excel (Microsoft, Inc., Redmond, WA, USA) and Tukey-Kramer’s multiple comparison test using SPSS 12.0J software (SPSS, Inc., Chicago, IL, USA).
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Disclosures

The authors have no conflicts of interest to declare.

References


Redox dynamics of chloroplast thiol enzymes


