Photosynthetic electron flow affects H$_2$O$_2$ signaling by inactivation of catalase in *Chlamydomonas reinhardtii*

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Abstract  A specific signaling role for H$_2$O$_2$ in *Chlamydomonas reinhardtii* was demonstrated by the definition of a promoter that specifically responded to this ROS. Expression of a nuclear-encoded reporter gene driven by this promoter was shown to depend not only on the level of exogenously added H$_2$O$_2$ but also on light. In the dark, the induction of the reporter gene by H$_2$O$_2$ was much lower than in the light. This lower induction was correlated with an accelerated disappearance of H$_2$O$_2$ from the culture medium in the dark. Due to a light-induced reduction in catalase activity, H$_2$O$_2$ levels in the light remained higher. Photosynthetic electron transport mediated the light-controlled down-regulation of the catalase activity since it was prevented by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosystem II. In the presence of light and DCMU, expression of the reporter gene was low while the addition of aminotriazole, a catalase inhibitor, led to a higher induction of the reporter gene by H$_2$O$_2$ in the dark. The role of photosynthetic electron transport and thioredoxin in this regulation was investigated by using mutants deficient in photosynthetic electron flow and by studying the correlation between NADP-malate dehydrogenase and catalase activities. It is proposed that, contrary to expectations, a controlled down-regulation of catalase activity occurs upon a shift of cells from dark to light. This down-regulation apparently is necessary to maintain a certain level of H$_2$O$_2$ required to activate H$_2$O$_2$-dependent signaling pathways.

Keywords  Catalase · *Chlamydomonas* · Hydrogen peroxide · Signaling · Thioredoxin

Abbreviations

APX  Ascorbate peroxidase
AT  Aminotriazole
DCMU  3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea
DTT  Dithiothreitol
MDH  Malate dehydrogenase
LUC  Luciferase
ROS  Reactive oxygen species
TRX  Thioredoxin

Introduction

Life in an oxygen-rich atmosphere has to cope with the danger of oxidative stress. Plants are particularly exposed to oxidative stress caused by photosynthetic processes. Reactive oxygen species (ROS) are produced during normal cell metabolism but their production is drastically enhanced when plants are exposed to stresses such as high light, low temperatures, or drought, or combinations thereof. Due to their high reactivity, ROS can damage
macromolecules essential for the integrity of the cell such as lipids, proteins, and nucleic acids. Thus, the concentration and accumulation of ROS needs to be strictly controlled. For this purpose, cells have evolved a variety of very efficient non-enzymatic (e.g., ascorbate, glutathione, tocopherols) and enzymatic (e.g., catalase, ascorbate peroxidase, superoxide dismutase, peroxiredoxins, thioredoxins, glutaredoxins) antioxidant systems (Apel and Hirt 2004; Foyer and Noctor 2005). The requirement for a control of ROS accumulation implicates that these reactive intermediates are sensed by cells in order to activate stress responses allowing adaptation to a new set of environmental conditions. It is now widely accepted that ROS, and especially H$_2$O$_2$, play a major role in cellular signaling pathways and regulation of gene expression in a wide variety of organisms including plants (Apel and Hirt 2004; Laloi et al. 2004). Several studies have used microarray analyses to study alterations in gene expression in response to ROS (Gadjev et al. 2006 and references therein). These studies have demonstrated that increased ROS levels alter the expression of a rather large set of genes (up to one-third of the genome). They also have shown that different ROS have specific signaling properties; a result supported by the recent discovery that H$_2$O$_2$ and singlet oxygen employ specific targets for the activation of an ROS-responsive promoter (Shao et al. 2007). The mechanisms that allow transduction of ROS signals to the nucleus remain unknown. In plants, the signaling pathways mediated by ROS involve heterotrimeric G-proteins (Joo et al. 2005) and protein phosphorylation mediated by MAP kinases and protein phosphatases (Kovtun et al. 2000; Gupta and Luan 2003; Rentel et al. 2004).

Under conditions of intracellular H$_2$O$_2$ production or treatment with exogenous H$_2$O$_2$, several plant H$_2$O$_2$ detoxifying enzymes, such as catalases and ascorbate peroxidases, are activated at the transcript, protein, and/or activity levels (Gechev et al. 2002; Vandenabeele et al. 2004; Yabuta et al. 2004; Davletova et al. 2005). Several studies have also shown that some of these enzymes may be inactivated by H$_2$O$_2$ (Miyake and Asada 1996; Shikanai et al. 1998; Hiner et al. 2000; Miyake et al. 2006).

Ascorbate peroxidases (APX) are the main H$_2$O$_2$ detoxifying enzymes in plants. Several isoforms of APX exist in plants and are located in the chloroplasts, the cytosol and peroxisomes (Shigeoka et al. 2002; Panchuk et al. 2005; Teixeira et al. 2006). Incubation of tobacco cell cultures in the presence of an H$_2$O$_2$ generating system (glucose + glucose oxidase) has been shown to result in inhibition of cytosolic APX activity (De Pinto et al. 2006). Also the endogenous generation of H$_2$O$_2$ via photosynthetic electron transfer resulted in inhibition of chloroplastic APX activity in tobacco plants (Miyake et al. 2006). APX were recently found among new putative targets of thioredoxins (TRX) identified by proteomic studies (Marchand et al. 2004; Wong et al. 2004; Yamazaki et al. 2004). Moreover, purified recombinant cytosolic APX from poplar was shown to be inhibited by reduced cytosolic thioredoxin h, reduced glutathione, and dithiothreitol. The molecular mechanism of this inhibition though remains to be determined (Gelhaye et al. 2006).

In plants, also catalases and peroxiredoxins play an important role in detoxifying H$_2$O$_2$ (for review see Feierabend 2005; Dietz et al. 2006). Light inactivation of catalase has been observed for enzymes of both plant and animal origin. Different isoforms of plant catalases show different sensitivities towards light (Grotjohann et al. 1997; Engel et al. 2006). The action spectrum of photo-inactivation is identical to the absorption spectrum of catalase, indicating that the light inactivation of catalases is caused by direct light absorption by the heme moieties. However, when irradiation was performed in the presence of chloroplasts the in vitro inactivation of catalase was enhanced. Under these conditions red light which is not absorbed by catalase but by photosynthetic pigments is sufficient to inhibit catalase (Feierabend and Engel 1986).

Catalases from *Chlamydomonas*, wheat, spinach, pea and potato were found among the potential targets of thioredoxin (TRX) identified by proteomic studies (Balmer et al. 2004; Lemaire et al. 2004; Maeda et al. 2004; Wong et al. 2004; Michelelet et al. 2006). TRX are small disulfide oxidoreductases which play a major role in light signaling and in the oxidative stress responses (Vieira Dos Santos and Rey 2006; Lemaire et al. 2007). We have previously shown that in total soluble extracts of *Chlamydomonas*, the activity of catalase could be modulated by cytosolic thioredoxin h: a 50% inhibition in catalase activity was observed after treatment with the cytosolic TRX system (NADPH, NADPH-thioredoxin reductase, TRX h) which could be mimicked by treatment with the strong reductant dithiothreitol (DTT) (Lemaire et al. 2004).

Hence, the question arises whether a down-regulation of the activity of ROS detoxifying enzymes, like APX and catalases, might be necessary to allow diffusion and perception of H$_2$O$_2$ under stress conditions that would result in the activation of genes that allow acclimation to changes in the environment. In order answer this question, we investigated whether photosynthetic electron transfer may be involved in the regulation of H$_2$O$_2$ detoxifying enzymes and thus the regulation of gene expression in response to H$_2$O$_2$. As a model system, we have chosen *Chlamydomonas reinhardtii* which was transformed with a construct containing a *Renilla reniformis* luciferase reporter gene under the control of an HSP70A promoter fragment that specifically responds to H$_2$O$_2$ (Shao et al. 2007).
Materials and methods

Algal strains and culture conditions

*Chlamydomonas reinhardtii* strains 325 (CW15, *arg7-8*) and D66 (CW15) were kindly provided by R. Matagne (University of Liège, Belgium) and Arthur Grossman (Carnegie Institute, Stanford, CA, USA), respectively. The wild-type strains CC-124 (mt–) and WT34 (mt+) (a derivative of strain 137c) were from the Chlamydomonas Genetics Center (Duke University, Durham, NC, USA) and the Institut de Biologie Physico-Chimique in Paris, respectively. Mutants *ApetD* (Kuras and Wollman 1994) and *rbcL* lacking both, the small and large subunit of Rubisco (rbcL-18-5B, Spritzer et al. 1989) were kindly provided by Olivier Vallon (IBPC, Paris). Cultures were grown photomixotrophically in Tris (rbcL-18-5B, Spreitzer et al. 1985) or maintained in the dark. When indicated, DCMU (*2 mM*) was added 45 min prior to illumination. The supernatants were mixed with an equal volume of 1 M KI. After 15 min at room temperature, the mixture was assayed for iodine formation by determining the OD at 390 nm. The absorbance at 390 nm was stable for at least 3 h (Waffenschmidt et al. 1993). Concentrations were determined using a standard calibration curve with known amounts of H₂O₂ (Sigma).

RNA isolation and RNA-blot analyses

RNA extraction, electrophoretic separation of RNA and hybridizations were performed as described previously (von Gromoff et al. 1989). The probe for *LUC* (accession number: AY004213) was a 0.9-kb *Bam HI*/XhoI fragment from pCrLUC (Fuhrmann et al. 2004). The *CBLP* gene encoding a *Chlamydomonas* Gβ-like polypeptide was employed as loading control gene (von Kampen et al. 1993).

Measurement of hydrogen peroxide

At each time point, 0.5-ml aliquots of the cultures were centrifuged at 13,000g in a microcentrifuge. The supernatants were mixed with an equal volume of 1 M KI. After 15 min at room temperature, the mixture was assayed for iodine formation by determining the OD at 390 nm. The absorbance at 390 nm was stable for at least 3 h (Waffenschmidt et al. 1993). Concentrations were determined using a standard calibration curve with known amounts of H₂O₂ (Sigma).

Measurement of enzyme activities

Cultures of *C. reinhardtii* strain D66 were grown to 1–2 × 10⁶ cells ml⁻¹, transferred in the dark for 16 h, and subsequently exposed to light (70 μmol photons m⁻² s⁻¹) or maintained in the dark. When indicated, DCMU (final concentration 6 μM) was added 45 min prior to illumination. Some cultures were supplemented with H₂O₂ (2 mM final concentration). Cells to be collected (1 × 10⁸ cells) were pelleted by centrifugation (microcentrifuge, maximal speed) and resuspended in 200 μl 50 mM Hepes buffer (pH 8). After two consecutive freeze/thawing cycles in liquid nitrogen, the enzyme activities in crude extracts of *Chlamydomonas* cultures were measured.
Catalase activity was measured polarographically at 20°C with a Clark-type electrode in 50 mM Hepes buffer (pH 8) in the presence of 1 mM H₂O₂ as substrate using a final protein concentration of 5 μg ml⁻¹. The protein concentration of the crude extracts was determined using the amido black assay.

Ascorbate peroxidase activity was measured photometrically at 290 nm in 20 mM phosphate buffer, pH 7.0, containing 0.5 mM ascorbate and 1 mM H₂O₂ as substrates using crude extract with a final protein concentration of 5 μg ml⁻¹.

Initial NADP-malate dehydrogenase activity was measured photometrically at 340 nm in 100 mM Tris/HCl, pH 7.9, containing 0.75 mM oxalacetate and 0.27 mM NADPH as substrates using crude extract with a final protein concentration of 10 μg ml⁻¹. To activate MDH to its maximal activity, samples were incubated with 10 mM DTT, 20-μM thioredoxin (cytosolic thioredoxin h1 from Chlamydomonas) in 100 mM Tris/HCl, pH 7.9 for 10 min prior to start of the assay by addition of substrates. The ratio between initial activity and total activity gives the percentage of activated MDH at the time point of harvest.

Statistics

Data represent means or representative examples from measurements repeated 3-6 times. Typical standard errors are shown, in Figs. 1b, 6 and 8; they are omitted for some data for clarity.

Results

Induction of an H₂O₂-responsive reporter gene by H₂O₂ is strongly stimulated by light

In the present work, we investigated the regulation of an H₂O₂-responsive reporter gene by exogenously added H₂O₂. For these assays we employed transformants harboring an H₂O₂-responsive fragment of the HSP70A promoter fused to a Renilla-derived luciferase gene (LUC) (Shao et al. 2007). A schematic representation of the LUC
reporter construct is presented in Fig. 1a. Transformants with this construct did not respond to heat shock but expression of the reporter gene is induced by hydrogen peroxide, either produced inside the chloroplast or added exogenously to the culture medium (Shao et al. 2007).

A strong increase in luciferase activity was observed in transformants with this construct after treatment by H$_2$O$_2$, but only when the cultures were incubated in the light (Fig. 1b). In the light, the activity increased about 200-fold within 5 h of incubation in the presence of 2 mM H$_2$O$_2$. In the dark, a distinctly weaker induction was observed. The luc activity was 11-fold higher after 3 h, and 19-fold higher after 5 h incubation. Light itself (70 μmol photons m$^{-2}$ s$^{-1}$) had no effect since no induction of the reporter gene could be observed in the light-grown control cultures supplemented with H$_2$O. In the absence of H$_2$O$_2$, no difference was observed between light-grown cultures and cultures transferred from the light to the dark. When cultures were grown in the dark for 20 h and then shifted to light in the absence of H$_2$O$_2$, a weak increase in luciferase activity was observed (Shao et al. 2007). This increase was 2.5- to 3-fold smaller than the one observed in the presence of H$_2$O$_2$ in the dark.

We next tested the level of expression in dependence on the concentration of H$_2$O$_2$ added to the culture medium. Luciferase activity was shown to increase strongly with the concentration of H$_2$O$_2$ as can be seen by plotting luciferase activity 3 and 5 h after H$_2$O$_2$ addition as a function of the concentration of externally added H$_2$O$_2$ (Fig. 1c). We have shown previously that much lower H$_2$O$_2$ concentrations are sufficient to induce the reporter gene when H$_2$O$_2$ is produced inside the chloroplast by the photosynthetic electron transfer chain in the presence of metronidazole as electron acceptor. In this case, a high level of induction of the reporter gene was observed already after 2 h, when the detectable H$_2$O$_2$ concentration in the medium was 3 μM (Shao et al. 2007).

Catalase activity contributes to a faster degradation of H$_2$O$_2$ in the dark.

The strong induction of transformants with the $P_{HSP70A-ASH1}$ -LUC reporter construct by externally added H$_2$O$_2$ observed only in the light may possibly be linked to lower rate of H$_2$O$_2$ degradation by Chlamydomonas cells incubated in the light than in the dark. This was tested by measuring the rate of H$_2$O$_2$ disappearance from the culture medium kept in light and or darkness. Indeed, H$_2$O$_2$ was more rapidly degraded by cultures in the dark than in the light (Fig. 2a). In the dark, H$_2$O$_2$ almost completely disappeared from the culture medium within 4 h and more than 50% of the initial H$_2$O$_2$ had disappeared already within 1 h. By contrast, in the light, about 75% of the H$_2$O$_2$ was still present after 1 h and some H$_2$O$_2$ could still be detected after 4 h. The slower disappearance of the H$_2$O$_2$ from the culture medium in the light may possibly be explained by the continuous production of H$_2$O$_2$ by the photosynthetic electron transport chain. However, the Mehler reaction as potential internal source of H$_2$O$_2$ cannot explain the difference between H$_2$O$_2$ degradation in the light and in the dark, because little H$_2$O$_2$ was produced by cultures, even in the presence of metronidazole, an artificial electron acceptor of PSI (less than 2 μM H$_2$O$_2$ per hour of irradiation was detectable in the medium; Shao et al. 2007).

![Fig. 2](image-url) a, b Degradation of exogenously added H$_2$O$_2$ by Chlamydomonas cultures. a Time course of degradation of H$_2$O$_2$ added to the medium of Chlamydomonas cultures in the light (open circles) or in the dark (closed circles). The change in H$_2$O$_2$ content in cell-free medium served as a control (open squares). 2 mM H$_2$O$_2$ was added at time point zero. Samples were taken at the times points indicated and the H$_2$O$_2$ concentration was determined as described in Sect. "Materials and methods". Light-grown C. reinhardtii cells were irradiated with 70 μmol photons m$^{-2}$ s$^{-1}$ of white light. b Time course of H$_2$O$_2$ degradation in the medium of Chlamydomonas cultures in the dark in the absence (closed circles) or in the presence of 2 mM aminotriazole, a catalase inhibitor (closed triangles). The inhibitor was added 45 min prior to the addition of 2 mM H$_2$O$_2$. All other conditions were as described in a.
This faster degradation of H$_2$O$_2$ in the dark may be caused by a light-dependent inhibition of H$_2$O$_2$ detoxifying enzymes. In plants, catalases and ascorbate peroxidases constitute the two major classes of enzymes involved in H$_2$O$_2$ detoxification. To test whether catalase is involved in the observed differences in H$_2$O$_2$ degradation between light and dark conditions, we measured the rates of H$_2$O$_2$ degradation in the dark in the presence or absence of the catalase inhibitor aminotriazole (AT; Fig. 2b). The concentration of H$_2$O$_2$ in the culture medium decreased considerably slower in the presence of AT than in its absence. This indicates that, under the conditions tested, catalase appears to be one of the major enzymes involved in H$_2$O$_2$ detoxification in *Chlamydomonas* cells. This result also suggested that a light-dependent regulation of catalase activity may account for the differences in H$_2$O$_2$ degradation observed between light and dark conditions (Fig. 2) and consequently for the light-dependence of the H$_2$O$_2$ inducibility of the reporter gene (Fig. 1b).

Light has previously been shown to inactivate plant catalases (Grotjohann et al. 1997; for review see Feierabend 2005). Generally, this inactivation was observed under high light conditions (800 µmol photons m$^{-2}$ s$^{-1}$). To test whether catalase activity was already affected by the low light intensities used in the experiments shown in Figs. 1 and 2, cell extracts of *Chlamydomonas* were illuminated with white light of an intensity of 70 µmol photons m$^{-2}$ s$^{-1}$ for up to 5 h. Under these conditions, no significant photoinactivation of the enzyme could be observed within 5 h of illumination (Fig. 6). We therefore concluded that a direct photoinactivation of catalase is unlikely to play a role under the low light conditions that were employed for studying the induction of the reporter gene by H$_2$O$_2$.

The redox state of the photosynthetic electron transfer chain affects H$_2$O$_2$ detoxification and catalase activity

Recently, it was shown that the activity of *Chlamydomonas* catalase is inhibited by reduced TRX in vitro (Lemaire et al. 2004). In chloroplasts, TRXs are reduced by the photosynthetic electron transfer chain in the light. The reduction of TRXs may thus possibly be responsible for the inactivation of catalases; and therefore, the slower H$_2$O$_2$ degradation observed in the light. Such a mechanism may account for the light dependence of the induction of the reporter gene by H$_2$O$_2$. In order to test whether photosynthetic electron transfer plays a role in this light dependence, we investigated the effect of DCMU, an inhibitor of photosystem II, on the expression of the reporter gene, the degradation of H$_2$O$_2$, and the activity of H$_2$O$_2$ detoxifying enzymes. In the presence of DCMU, linear photosynthetic electron transfer is blocked and, among other components which may play a role in redox regulation, chloroplastic TRXs remain oxidized. In the presence of DCMU, only a small increase in luciferase activity could be detected after treatment with H$_2$O$_2$ in the light (Fig. 3), indicating only a weak induction of the reporter gene. The level of luciferase activity observed was similar to that seen after addition of H$_2$O$_2$ in the dark. This suggests that linear photosynthetic electron flow is required for an efficient induction of the reporter gene by H$_2$O$_2$ in the light.

If this requirement is linked to a light-dependent inactivation of catalase, implicating TRXs or other mediators, then DCMU was predicted to affect the rate of H$_2$O$_2$ degradation in the light and the activity of catalases. Therefore, the time course of H$_2$O$_2$ consumption was measured in the light in the presence and absence of DCMU (Fig. 4). In the light, the H$_2$O$_2$ concentration decreased significantly faster in the presence of DCMU than in its absence. This suggests that photosynthetic electron flow accounts for the effect of light on the rate of H$_2$O$_2$ degradation in the culture medium.

To investigate the effect of the redox state of photosynthetic electron flow further, the degradation of H$_2$O$_2$ was tested using mutants lacking either the cytochrome b$_6$f complex (*petD*) or deficient in ribulose-1,5-bisphosphate carboxylase (Rubisco) (*rbcL*). As shown in Fig. 5, only a small difference in the kinetics of H$_2$O$_2$ degradation was observed between dark-treated and light-treated cultures of the *petD* mutant lacking the cytochrome b$_6$f complex. The differences were statistically not significant according to the Student's *t* test. In this mutant, forward electron transport to PSI is completely abolished, the plastoquinone pool stays...
The activity could be inhibited by 75% by addition of 5-mM DTT (Fig. 6). This indicates that the catalase can undergo redox dependent inactivation. Figure 6 shows that incubation of the cells in the light clearly inhibited the activity of catalase. Indeed, about 40% of the activity was lost within 2 h and only 25% remained after 5 h of incubation in the light. The remaining activity after 5 h illumination corresponded to the activity of the catalase in the presence of DTT. By contrast, in cells maintained in the dark, the activity remained above 85% of the initial activity throughout the experiment. Presence of DCMU during the light incubation protected against the loss of activity (Fig. 6), supporting the idea that the activity of H$_2$O$_2$-consuming enzymes is regulated via the redox state of the electron transfer chain. The kinetics of inactivation were not dependent on the presence of H$_2$O$_2$ in the culture medium, since the same degree of inhibition was observed when no H$_2$O$_2$ was added prior to the shift of the cultures from dark to light (data not shown).

The activity of ascorbate peroxidases did not show any inhibition during the time course of illumination of the cultures in the presence of 2 mM H$_2$O$_2$ (Fig. 7), indicating that indeed the redox-dependent inhibition of catalase is the important factor leading to a loss of H$_2$O$_2$ degradation and subsequent expression of the reporter gene. To show a correlation between the state of reduction of thioredoxins and the activity of catalase, we measured the activity of the NADP-dependent malate dehydrogenase (MDH), one of the classical enzymes activated by reduction of its disulfide bonds by reduced thioredoxin (e.g., Lemaire et al. 2005, 2007). MDH activity increased upon incubation in the light while catalase activity decreased (Fig. 7). These data support the hypothesis that the activity of catalase is

![Fig. 4](image-url) Effect of DCMU on the degradation of H$_2$O$_2$ in the medium by Chlamydomonas cells. Cells were incubated with irradiation (white light, 70 μmol photons m$^{-2}$ s$^{-1}$) in the presence of 6 μM DCMU for 45 min (open triangles) or its absence (open circles) prior to addition of 2 mM H$_2$O$_2$. Results from cells incubated in the absence of DCMU in the dark (closed circles) are also shown. Samples were taken at the times points indicated and the H$_2$O$_2$ concentration in the media was determined as described in Sect. “Materials and methods.”

![Fig. 5](image-url) Degradation of exogenously added H$_2$O$_2$ in the medium by photosynthesis mutants. Time course of degradation of H$_2$O$_2$ added to the medium of Chlamydomonas cultures in the light (white light, 55 μmol photons m$^{-2}$ s$^{-1}$) (open symbol) or in the dark (closed symbol). The rbcL and ΔpetD mutants were grown in very dim light (<10 μmol photons m$^{-2}$ s$^{-1}$) for 5 days and then diluted to 1 x 10$^8$ cell ml$^{-1}$. The parental strain of the mutants (WTS34), named WT, was incubated with irradiation (white light, 55 μmol photons m$^{-2}$ s$^{-1}$) for 3 days and then diluted to 1 x 10$^6$ cell ml$^{-1}$. Right after dilution, H$_2$O$_2$ (final concentration 2 mM) was added. Samples were taken at the times points indicated and the H$_2$O$_2$ concentration in the media was determined as described in Sect. “Materials and methods.”
linked to the redox state of thioredoxin in the chloroplast. 

DCMU inhibited the activation of MDH as expected (Fig. 7).

It has to be mentioned that H$_2$O$_2$ inhibits CO$_2$ fixation, since the transketolase reactions of the Calvin cycle are very sensitive to inhibition by H$_2$O$_2$ (Kaiser 1976). Under our conditions, photosynthetic oxygen evolution measured in the presence of 1 mM NaHCO$_3$ was inhibited by 50% upon addition of 2 mM H$_2$O$_2$ (data not shown) in accordance with data reported for a different green algal species (Drabokova et al. 2007). This effect, however, seems not to interfere with our observations, neither with the induction of the reporter gene nor with the light-dependent inactivation of catalase.

Aminotriazole and DCMU affect the expression of the reporter gene at the mRNA level

We have shown above that a down-regulation of catalase activity mediated either by photosynthetic electron flow or the addition of AT appears to be responsible for a slower degradation of H$_2$O$_2$ in the light. This suggested that a down-regulation of catalase activity in the light also accounts for the light dependence of the LUC reporter gene induction by H$_2$O$_2$. In order to confirm that the activity of H$_2$O$_2$ degrading enzymes and photosynthetic electron flow indeed are involved in the regulation of the reporter gene and to rule out the possibility that DCMU and AT interfere with luciferase activity directly, we measured the effect of AT and DCMU on H$_2$O$_2$ induction of the reporter at the mRNA level (Fig. 8). In the light, an increase of LUC mRNA level was observed after H$_2$O$_2$ treatment. This induction was abolished by the presence of DCMU, confirming that photosynthetic electron flow is required for induction of the reporter gene, as already observed at the activity level (Fig. 3). In the dark, a small increase in LUC mRNA levels was observed upon H$_2$O$_2$ addition, consistent with the low level increase of LUC activity observed under these conditions (Fig. 1b). Addition of AT in the dark allowed a stronger increase in LUC mRNA levels after H$_2$O$_2$ treatment, consistent with a lower degradation of this ROS (Fig. 2b). These results are in agreement with those observed at the LUC activity level and indicate that down-regulation of the activity of H$_2$O$_2$ degrading enzymes is a prerequisite for an efficient induction of the reporter gene by H$_2$O$_2$. 

![Fig. 6](image1.png) Effect of light and DCMU on the activity of catalase measured in vitro. Cells were incubated in the presence of 2 mM H$_2$O$_2$ in the light (white light, 70 μmol photons m$^{-2}$ s$^{-1}$) in the absence (circles) or presence of 6 μM DCMU (open triangles) and in the dark without DCMU (closed circles) for the time indicated. H$_2$O$_2$ and DCMU were added at time point zero. Cells were harvested at the time indicated and opened up by freeze/thawing. H$_2$O$_2$ consumption by the crude extracts was measured polarographically. 100% activity corresponds to an activity of 10 μmol O$_2$ mg protein$^{-1}$ min$^{-1}$ produced when 1 mM H$_2$O$_2$ was added as substrate. The catalase activity in the crude extract of cells incubated in the light was strongly inhibited when 5 mM DTT was added (stars). Also shown is the catalase activity of crude extract of dark-grown cells (diamonds) that were illuminated (white light, 70 μmol photons m$^{-2}$ s$^{-1}$) for the time indicated

![Fig. 7](image2.png) Effect of light on the activity of ascorbate peroxidase and on the activity of NADP-malate dehydrogenase (MDH) in vitro. Cells were incubated in the presence of 2 mM H$_2$O$_2$ in the light (white light, 70 μmol photons m$^{-2}$ s$^{-1}$) for the time indicated. H$_2$O$_2$ and DCMU were added at time point zero. Cells were harvested at the time indicated and opened up by freeze/thawing. Ascorbate peroxidase (squares), activity was measured photometrically at 290 nm in the crude extract in the presence of ascorbate and H$_2$O$_2$ as outlined in Sect. “Materials and methods”. 100% activity corresponds to an activity of 18 μmol dehydroascorbate mg protein$^{-1}$ min$^{-1}$ produced when 1 mM H$_2$O$_2$ was added as substrate. The activity of MDH was measured photometrically at 340 nm in the presence of NADPH and oxalacetate in the absence (inverted triangles) and in the presence of 6 μM DCMU (diamonds). Maximal activity (100%) was achieved by incubation of samples with DTT and thioredoxin h (see Sect. “Materials and methods” for details”). For comparison the catalase activity (circles) is shown (data taken from Fig. 6)
Fig. 8 Effect of aminotriazole and DCMU on the H$_2$O$_2$ induction of the $P_{{\text{RPNA}}}$-LUC reporter gene at the RNA level in Chlamydomonas transformants. Cultures incubated in the dark (D) were treated with H$_2$O$_2$ (2 mM final concentration) in the presence (lane 4) or absence (lane 5) of the catalase inhibitor aminotriazole (2 mM AT). AT was added 45 min prior to the addition of H$_2$O$_2$. Cells incubated in the light (L) were treated with H$_2$O$_2$ (2 mM final concentration) in the presence (lane 2) or absence (lane 1) of the PSI inhibitor DCMU (6 μM). DCMU was added 45 min prior to the addition of H$_2$O$_2$. A control culture that did not receive H$_2$O$_2$ was incubated in the light (lane 1). Samples for RNA isolation were taken 2 h after H$_2$O$_2$ addition. RNA blots (10 μg total RNA per lane) were hybridized with a probe specific for the LUC transgene and CBLP, the latter serving as a loading control as described in Sect. “Materials and methods”

Discussion

The data presented here show that the expression of the H$_2$O$_2$-responsive reporter construct not only depended on the H$_2$O$_2$ concentration in the media (Fig. 1c) but also on the activity state of H$_2$O$_2$ degrading enzymes (Figs. 2, 3, 8). The decrease in the activity of H$_2$O$_2$ degrading enzymes observed upon illumination was prevented by DCMU (Fig. 4) and by mutations perturbing photosynthetic electron flow (Fig. 5), indicating that catalase activity was correlated with the redox state of the photosynthetic electron transport chain (Fig. 6). These data illustrate that Chlamydomonas cells have evolved mechanisms that ensure maintenance of a certain level of H$_2$O$_2$ in order to allow activation of H$_2$O$_2$-responsive genes. In photosynthetic organisms, ROS are mainly formed in the light as a consequence of electron transport reactions under conditions where the electron flow system is saturated and the electron acceptors are reduced. In the so-called Mehler reaction, the electron acceptors of photosystem I react with oxygen when the ferredoxin-NADP system and alternative electron sinks are already reduced. Superoxide anions and H$_2$O$_2$ are formed. These ROS are released into the stroma where they encounter an efficient antioxidant system and detoxifying enzymes (for review see Asada 1999; Foyer and Noctor 2005). In general, it is assumed that detoxifying enzymes have to be present in an active state during oxidative stress conditions.

However, it was recently observed that higher plant ascorbate peroxidase is inhibited in the light under conditions where both, the Calvin cycle and photorespiration, are inhibited (Miyake et al. 2006). This paradoxical situation makes sense when the function of ROS in gene regulation is taken into account. Maintenance of a certain H$_2$O$_2$ concentration inside the chloroplast may be achieved in higher plants via control of the activity of ascorbate peroxidases, the main H$_2$O$_2$-detoxifying enzyme in this organelle. The H$_2$O$_2$ concentration in the cell will depend on the activity of the whole set of detoxifying enzymes; also on those located in different organelles because H$_2$O$_2$ can easily diffuse across biological membranes (Bienert et al. 2007). As shown for different organisms, the expression of a number of genes, encoding, e.g., heat shock proteins, catalases, and ascorbate peroxidases involved in the abrogation of oxidative stress, is regulated via H$_2$O$_2$ signaling. Thus, it can be postulated that a down-regulation of the activity of detoxifying enzymes under stress conditions is a necessary prerequisite for the activation of those genes that allow acclimation of the organism to oxidative stress conditions. In Chlamydomonas, this may be achieved via inactivation of catalase; the activity of ascorbate peroxidase under these conditions being constant (Fig. 7). Inactivation of plant catalases by high intensity irradiation (Feierabend and Engel 1986; Grotjohann et al. 1997) needs to be distinguished from low light induced inactivation as studied here by exposing Chlamydomonas cells to light of an intensity of 70 μmol photons m$^{-2}$ s$^{-1}$ or less. It has also been reported previously that the catalase activity in Chlamydomonas is higher in cells grown in the dark than in the light (Kato et al. 1997).

The inactivation of H$_2$O$_2$ degrading enzymes by light of moderate intensity as observed in Chlamydomonas could be linked to photosynthetic electron transfer since this inactivation was prevented by DCMU known to block the photosynthetic apparatus at PSII (Fig. 4). In vitro measurements revealed that catalase is the target for this inactivation (Fig. 6). The redox sensor which mediates the inactivation of H$_2$O$_2$ degrading enzymes appears to be located downstream from PSI, since a mutation leading to an inactivation of the cytochrome b$_{6f}$ complex (ApetD) resulted in a protection against inhibition of the activity of H$_2$O$_2$ degrading enzymes by light (Fig. 5). The reduction state of the plastoquinone pool (Pfannschmidt and Liere 2005) can thus be ruled out to be of importance in the regulation of catalase activities, because mutations leading to an inactivation of the cytochrome b$_{6f}$ complex are characterized by a high reduction state of the plastoquinone pool while in the presence of DCMU, the plastoquinone pool stays oxidized. Since a reduction of TRX by the photosyn-
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In the genomic sequence of *Chlamydomonas* (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html), only a single gene encoding a typical catalase (gene model Chlre3/scaffold_30:1312932-1317626) was observed. A gene (gene model Chlre3/scaffold_1:4550894-455823) encoding a putative catalase–peroxidase homologous to those found in prokaryotes (Jakopitsch et al. 2005) was also found. However, as judged on the basis of the number of EST sequences, expression of the latter gene appears to be very weak as compared to the expression of the typical catalase gene, even under oxidative stress conditions. Consequently, the catalase activities reported here are likely to correspond to the activity of the typical catalase, although it cannot be excluded that the putative catalase–peroxidase also contributes to \( \text{H}_2\text{O}_2 \) degrading activity measured in extracts.

In higher plants, catalases were localized to peroxisomes (Heazlewood et al. 2005), while in *Chlamydomonas* the enzyme is most likely located in mitochondria (Kato et al. 1997; Lemaire et al. 2004). This implies that a signal about the redox state of the photosynthetic electron transfer chain has to be transmitted to targets outside of the chloroplast. This signal could be initiated by a component whose redox state depends on the redox state of a functional photosynthetic electron transfer chain such as TRXs. The signal is possibly transmitted from the chloroplast by redox mediators such as glutathione or NADPH. Indeed, reducing equivalents of chloroplastic NADPH have been reported to be transferred outside of the chloroplast in the form of malate (Scheibe 2004). This malate is formed by reduction of oxalacetate using NADPH as electron donor. Interestingly, the NADP-malate dehydrogenase of the chloroplast is activated by light by a mechanism dependent on the redox state of plastidic TRXs (Lemaire et al. 2005, 2007). Indeed, a reverse correlation between the activities of catalase and NADP-malate dehydrogenase was observed (Fig. 7). Once outside of the chloroplast, the signal may be transduced to a redox regulator of the catalase via different redox mediators. Since the activity of *Chlamydomonas* catalase has been shown to be regulated by TRXs in vitro (Lemaire et al. 2004), the redox regulator of catalase is likely to be a TRX or involves TRX-related proteins like glutaredoxins. There is some evidence that thioredoxins belonging to the TRX o and TRX h subtypes are present in the mitochondria of *Chlamydomonas* (Lemaire et al. 2003).

In conclusion, the data presented provide support for the idea that *Chlamydomonas* has evolved mechanisms that allow maintenance of a certain level of \( \text{H}_2\text{O}_2 \). We assume that these mechanisms ensure a regulation of gene expression in response to ROS, allowing for an adaptation of the organisms to stress conditions. A fast inactivation of catalase activity is seen when cells are exposed to a 10-fold higher light intensity (data not shown). Regulation of the activity of ROS detoxifying enzymes appears to play an important role. A regulation via TRX or other components of the regulatory redox network like glutaredoxins, peroxiredoxins or glutathione provides an attractive strategy for the regulation of such enzyme activities. These compounds may ensure a fast and reversible inactivation of the enzymes, thereby allowing for a rapid sensing of changes in environmental conditions. Most importantly, through thioredoxins or other redox compounds, the regulation of the activity of \( \text{H}_2\text{O}_2 \)-consuming enzymes may directly be coupled to photosynthetic electron flow, and thus to the main source for ROS production in photosynthetic organisms.

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