Plant, Cell & Environment

Plant, Cell and Environment (2013) **36**, 1204–1213 doi: 10.1111/pce.12053

Down-regulation of catalase activity allows transient accumulation of a hydrogen peroxide signal in *Chlamydomonas reinhardtii*

LAURE MICHELET[1,*], THOMAS ROACH[1], BEAT B. FISCHER[2], MARIETTE BEDHOMME[3], STÉPHANE D. LEMAIRE[3] & ANJA KRIEGER-LISZKAY[1]

[1]*Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA) Saclay, Institut de Biologie et Technologie de Saclay, Centre National de la Recherche Scientifique, UMR 8221, 91191 Gif-sur-Yvette Cedex, France,* [2]*Department of Environmental Toxicology, Eawag, Swiss Federal Institute of Aquatic Science & Technology, Ueberlandstrasse 133, CH-8600 Dübendorf, Switzerland and* [3]*Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, FRE3354 Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France*

ABSTRACT

In photosynthetic organisms, excess light is a stress that induces production of reactive oxygen species inside the chloroplasts. As a response, the capacity of antioxidative defence mechanisms increases. However, when cells of *Chlamydomonas reinhardtii* were shifted from dark to high light, a reversible partial inactivation of catalase activity was observed, which correlated with a transient increase in the level of H_2O_2 in the 10 μM range. This concentration range seems to be necessary to activate H_2O_2-dependent signalling pathways stimulating the expression of H_2O_2 responsive genes, such as the heat shock protein *HSP22C*. Catalase knock-down mutants had lost the transient accumulation of H_2O_2, suggesting that a decrease in catalase activity was the key element for establishing a transient H_2O_2 burst. Catalase was inactivated by a one-electron event consistent with the reduction of a single cysteine. We propose that under high light intensity, the redox state of the photosynthetic electron transport chain is sensed and transmitted to the cytosol to regulate the catalase activity. This allows a transient accumulation of H_2O_2, inducing a signalling event that is transmitted to the nucleus to modulate the expression of chloroplast-directed protection enzymes.

Key-words: EPR spin trapping; signalling.

INTRODUCTION

Life in an oxygenic atmosphere unavoidably leads to the production of toxic reactive oxygen species (ROS) during normal cell metabolism, which increases under stress (Apel & Hirt 2004). Aerobic organisms have developed a set of defence mechanisms involving antioxidant molecules such as tocopherols, glutathione, ascorbate and enzymes such as superoxide dismutase, peroxiredoxins, glutathione peroxidase, ascorbate peroxidase (APX) and catalase to combat ROS (Noctor, Veljovic-Jovanovic & Foyer 2000; Apel & Hirt 2004; Mene-Saffrane, Jones & DellaPenna 2010; Mhamdi *et al*. 2010; Dietz 2011). However, it is recognized that ROS also act as signalling molecules that can trigger cell responses (op den Camp *et al*. 2003; Fischer, Krieger-Liszkay & Eggen 2005; Gadjev *et al*. 2006; Laloi *et al*. 2007; Bechtold *et al*. 2008; Foyer & Noctor 2011; Gough & Cotter 2011; Mittler *et al*. 2011; Queval *et al*. 2012). In this context, focus has centred on H_2O_2, the most stable ROS. H_2O_2 generated in chloroplasts during the light phase is able to diffuse out into the cytosol (Mubarakshina *et al*. 2010) where it can function directly as a signalling agent. To act in this function, H_2O_2 must be able to rise rapidly to a threshold concentration and remain high enough (10–100 μM) for a sufficient time so that it can oxidize the molecules involved in the cell signalling events (Stone & Yang 2006). Therefore, enzymes that metabolize ROS must play a dual role: in an active state, they keep ROS concentrations at safe levels, and in a deactivated state, they can allow internal ROS concentrations to reach critical levels for activation of signalling components.

In mammalian cells, the 'floodgate hypothesis' (Wood, Poole & Karplus 2003) posits that an overoxidation of the peroxidatic cysteine of 2-Cys peroxiredoxin (Prx) to the sulfinate form, and its subsequent slow reduction by sulfiredoxin (Biteau, Labarre & Toledano 2003; Woo *et al*. 2003), leads to a peak in H_2O_2 that allows this ROS to take on a signalling role. Furthermore, in the case of receptor signalling, a membrane-associated 2-Cys Prx has been recently shown to be reversibly inactivated by phosphorylation in stimulated cells (Woo *et al*. 2010). This localized inactivation of 2-Cys Prx likely allows generation of favourable H_2O_2 gradients at localized regions across membranes, where signalling proteins are concentrated, while minimizing the general accumulation of H_2O_2 to toxic levels.

In plants, the role of a transient accumulation of ROS during light stress has yet to be clearly addressed. In the

Correspondence: A. Krieger-Liszkay. Fax: +33 16908 8717; e-mail: anja.krieger-liszkay@cea.fr

*Present address: Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, FRE3354 Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France.

green alga *Chlamydomonas reinhardtii*, exogenously added H_2O_2 provokes a different response in the light than in the dark (Shao *et al*. 2008). When added to a dark-adapted culture, H_2O_2 was efficiently detoxified and did not lead to expression of an H_2O_2-specific reporter gene. By contrast, when added during illumination, H_2O_2 detoxification was much less efficient, allowing expression of the H_2O_2-specific reporter gene. This differential response was due to a lower catalase activity in the light. In this previous study, *C. reinhardtii* was shifted from dark to low-intensity growth light for several hours, and non-physiological concentrations of H_2O_2 (2 mM) were added to the cultures. The physiological importance of such inactivation of catalase has not been investigated so far.

Here, we present data showing that the previously observed inactivation of catalase activity occurs rapidly upon exposure of the cells to high light (HL) in the absence of exogenously added H_2O_2. In response to HL, we observed a transient accumulation of H_2O_2 during the first 10 min, which was concomitant with a decrease of catalase activity. The accumulation of H_2O_2 in the medium was measured by a spin-trapping assay. This transient H_2O_2 accumulation was absent in catalase knock-down mutants. The effects we observed strongly support the hypothesis that catalase activity is regulated upon exposure to HL to allow a transient accumulation of H_2O_2 for signalling.

MATERIALS AND METHODS

Cultures

Cell-wall-less green alga *C. reinhardtii* D66 (referred to as WT) were grown in Tris–acetate–phosphate (TAP) medium to densities of $1–5 \times 10^6$ cells mL^{-1} under light–dark cycles (16 h, 70 μmol quanta m^{-2} s^{-1}/8 h dark) at 25 °C. HL treatment (white light, 700 μmol quanta m^{-2} s^{-1}) was started after 8 h dark.

Recombinant catalase

Full-length catalase cDNA (CAT1, A8J537, UNIPROT) was used for cloning between the NdeI and BamHI sites of the pET-3c-His vector by standard molecular biology methods. Recombinant catalase was expressed in *Escherichia coli* BL21 grown at 18 °C for 18 h. After re-suspension in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–sodium hydroxide (HEPES-NaOH; pH 8), 500 mM NaCl, 10% glycerol, 10 mM imidazole, 100 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Complete Mini, Roche Applied Science, Basel, Switzerland), cells were broken by three passages in a French Press (7×10^7 Pa). After centrifugation, catalase in the supernatant was purified by Ni^{2+} affinity chromatography.

RNA extraction and quantitative PCR analysis

A total of 50 mL of culture [$(1–2) \times 10^6$ cells mL^{-1}] were harvested by centrifugation and the pellets were stored in liquid nitrogen. Furthermore, 1 mL of Tri Reagent® (Sigma-Aldrich, St Louis, MO, USA) and 500 μL of glass beads (G8772; Sigma-Aldrich) were added to the frozen pellets, and the suspensions were vigorously agitated for 2 min. The homogenates were transferred to a Phase Lock Gel™ tube (5 PRIME, Hamburg, Germany). After 1 min of incubation at 20 °C, 200 μL of chloroform was added before thorough mixing. After 10 min of centrifugation at 14.000 *g* at 4 °C, the RNA was precipitated from the aqueous phase with 0.5 mL of isopropanol, collected by centrifugation and washed with 70% ethanol.

After purification, RNA was quantified spectrophotometrically and quality was assessed by agarose gel electrophoresis. For qPCR experiments, 300 ng of individual total RNA was used in each 15 μL reverse transcription reaction with a reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. Sequences of primers for qPCR were designed with the Primer Express™ software (Applied Biosystems) and qPCR reactions were performed on the ABI Prism® 7500 Sequence Detection System using the SYBR® Green technology (Applied Biosystems), as described in Fischer *et al*. (2005). The C_t values of the *CBLP* gene was used for normalization of variable RNA levels. Relative expression was calculated for each treatment compared to the untreated control as an average with standard error (SEM) out of three independent experiments.

Generation of catalase knock-down mutants

The Web MicroRNA Designer platform (WMD3, http://wmd3.weigelworld.org) suggested suitable amiRNA candidates to silence *Chlamydomonas* catalase gene expression (CAT1, A8J537, UNIPROT). The amiRNA constructs were generated following the 'dsDNA Oligonucleotide cloning for amiRNA Expression' protocol (Molnar *et al*. 2009), using the pChlamiRNA3int vector and the following primers:

amiFor_cat:
5′ctagtGAGACGTATGTCAAGTACCAAtctcgctgatcggcac catgggggtggtggtgatcagcgctaTTGGAACTTGACATACGTC TCg3′

amiRev_cat:
5′ctagcGAGACGTATGTCAAGTTCCAAtagcgctgatcacca ccacccccatggtgccgatcagcgagaTTGGTACTTGACATACGT CTCa3′

pChlamiRNA3int_cat was used to transform the D66 *Chlamydomonas* strain and transformants were selected on TAP 1.6% agar plates supplemented with 15 μg mL^{-1} paromomycin. Transformants with decreased catalase activity compared to the parental strain were identified by testing their sensitivity towards H_2O_2 in growth tests and further confirmed by catalase activity assays in total soluble extracts. Catalase levels were monitored by Western blot using anti-catalase antibodies raised in rabbits by Genecust (Dudelange, Luxembourg) against the peptide 'MDPAKIRPSSAYNTPY' from *Chlamydomonas* catalase sequence.

Growth test: H_2O_2 *sensitivity test*

For spot test analysis, *Chlamydomonas* strains were inoculated in TAP liquid medium and were grown to exponential phase [(1–2) × 10^6 cells mL^{-1}]. After measurement of catalase activity in the different cultures, 10 μL drops of the different liquid cultures adjusted at 1 × 10^6 cells mL^{-1} was spotted on TAP 1.6% agar Petri dishes supplemented with 0, 0.4 or 0.8 mM H_2O_2 added after the agar had cooled, but before it had set.

Measurement of enzyme activities

Catalase and APX activities were measured as in Shao *et al.* (2008). For APX activity assays, 1 mM ascorbate was added to the samples before freezing. Superoxide dismutase (SOD) activity was measured photometrically at 470 nm using xanthine/xanthine oxidase as $O_2^{\bullet-}$ generating system and XTT (Na,3′-(1-(phenylamino-carbonyl)-3,4-tetrazolium)-bis-(4-methoxy-6-nitro)benzene sulfonic acid hydrate) for the detection of $O_2^{\bullet-}$ ($\varepsilon_{XTT,470}$ = 24.2 mM^{-1} cm^{-1}). The assay contained 50 μM xanthine, 100 μM XTT and 0.2 U mL^{-1} xanthine oxidase in 20 mM HEPES (pH 7.0). SOD activity was determined by following the inhibition of the $O_2^{\bullet-}$ production after the addition of 100 μg protein mL^{-1} of crude extract.

Measurements of ascorbate and free-thiol content

Ascorbate content

Ascorbate was measured using a colorimetric assay (Kampfenkel, Van Montagu & Inze 1995), which relies on the reduction of Fe^{3+} ions to Fe^{2+} by ascorbate. A total of 10 mL of *Chlamydomonas* culture (~10 μg chl mL^{-1}) was centrifuged at 13 000 *g* for 3 min at 23 °C. The pellet was re-suspended in 0.1 M HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and immediately frozen in liquid N_2. The sample was thawed, briefly vortexed and then centrifuged at 13 000 *g* for 3 min at 3 °C. For the measurement of total ascorbate, 50 μL of the supernatant was incubated at 21 °C with 5 mM dithiothreitol (DTT) in 50 μL of 0.4 M potassium phosphate buffer, pH 7.4 (P-buffer). After 15 min, 25 μL of 0.5% (w/v) *N*-ethylmaleimide (NEM) was added and then the sample was incubated at 42 °C for 45 min, with 200 μL of working reagent. For the measurement of reduced ascorbate, the samples were incubated as for total ascorbate but without DTT or NEM. The working reagent consisted of reagent A [4.6% (w/v) trichloroacetic acid, 15.3% (v/v) orthophosphoric acid (H_3PO_4), 0.6% (w/v) iron (III) chloride] and reagent B [4% (w/v) 2,2′-dipyridyl in 70% (v/v) ethanol] combined in a 11:4 (v/v) ratio, and absorbance was measured at 520 nm. Ascorbate was calculated against separate standard curves following the same treatments for total or reduced ascorbate.

Free-reduced thiols

Reduced thiols were measured using a colorimetric assay with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (Ellman's reagent) according to Rahman, Kode & Biswas (2006), with modifications as stated below. A total of 500 μL *Chlamydomonas* culture was diluted with 500 μL of 10% trichloroacetic acid, centrifuged at 13 000 *g* for 3 min at 4 °C, and the supernatant was collected. For the assay, 200 μL of the supernatant was diluted in 0.5 M phosphate buffer (pH 7.5) and 5 mM DTNB, incubated for 3 min at room temperature, and the absorption was measured at 412 nm (ε_{TNB} = 13 600 M^{-1} cm^{-1}).

Electron paramagnetic resonance (EPR) spectroscopy

Spin-trapping assays were performed with 4-pyridyl-1-oxide-*N-tert*-butylnitrone (4-POBN). Samples were collected at the given time points and incubated for 5 min in the presence of 50 mM 4-POBN, 4% ethanol and 50 μM Fe-EDTA. EPR spectra were recorded at 20 °C using an e-scan spectrometer (Bruker, Rheinstetten, Germany). Used parameters were as follows: microwave frequency, 86 GHz; modulation amplitude, 1 G; microwave power, 14 mW; receiver gain, 2 × 10^3; time constant, 5.1 ms; number of scans, 4.

Redox titration

Catalase (5 μg mL^{-1}) was incubated for 1.5 h in 5 mM dithiothreitol (DTT_{red})/*trans*-4,5-dihydroxy-1,2-dithiane (DTT_{ox}) in different ratios in 50 mM HEPES (pH 8.0) at 20 °C. The redox potential of the medium was calculated according to the Nernst equation. Trp fluorescence was excited at 297 nm, and the emission was measured between 310 and 400 nm. The same incubation time was used for measuring the dependency of catalase activity on the redox potential.

Polyacrylamide gel electrophoresis (PAGE)

Native PAGE was carried out in 8% gels containing 0.1% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The sample buffer contained 40 mM Tris–HCl (pH 7.8), 0.1% CHAPS, 10% glycerol and 0.002% bromophenol blue. The running buffer contained 25 mM Tris, 1.44% Gly and no detergent. Catalase activity was detected in gel by negative staining, by incubating the gel in 1 mM H_2O_2 for 15 min before adding 1% $FeCl_3$ and 1% $K_3[Fe(CN)_6]$.

RESULTS

Characterization of wild-type *C. reinhardtii* response to HL stress

The amount of H_2O_2 accumulated in *C. reinhardtii* at the given time points after transfer from dark to HL were determined by a spin-trapping assay containing 4-POBN/ethanol/Fe-EDTA (Fig. 1a,b). 4-POBN/ethanol reacts with hydroxyl radicals, leading to the formation of a stable organic radical that can be detected by EPR spectroscopy. The $^{\bullet}OH$ radicals originate from the so-called Fenton reaction between H_2O_2 generated by *C. reinhardtii* and the added ferrous Fe-EDTA.

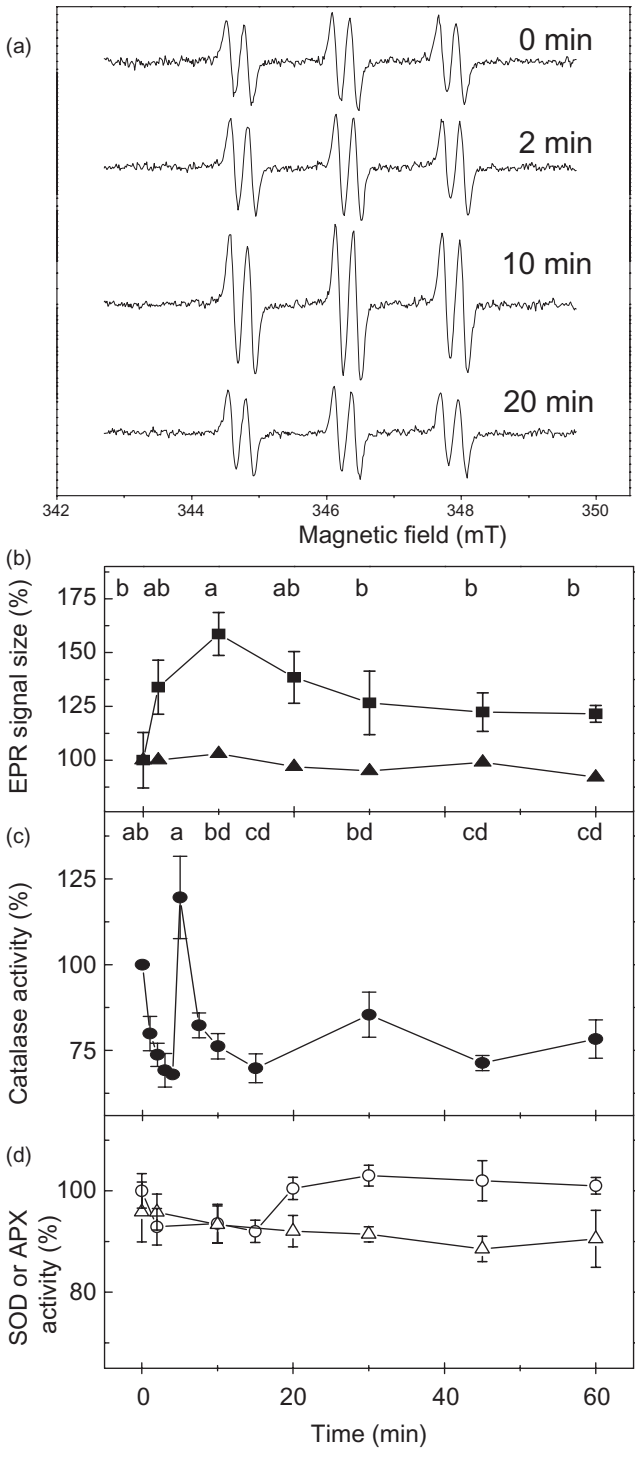

Figure 1. Transient accumulation of H_2O_2 after a dark to high light (HL) shift in wild-type *Chlamydomonas reinhardtii*. Cells were exposed after 8 h dark to HL (700 μmol quanta m^{-2} s^{-1}) for 60 min. (a) Typical electron paramagnetic resonance (EPR) spectra of the 4-POBN/α-hydroxyethyl adduct recorded at 0, 2, 10 and 20 min after the dark to HL shift. The size of the EPR spectra indicates the concentration of H_2O_2 indirectly by the trapping of hydroxyl radical ($HO^{\bullet}$). $HO^{\bullet}$ was generated from H_2O_2 in a Fe^{2+}-catalysed Fenton reaction. The amplitude of the EPR signal recorded is proportional to the amount of H_2O_2 present in the samples (Supporting Information Fig. S1) and corresponds to approximately 30 μmol H_2O_2 mg Chl^{-1} h^{-1} during the first 10 min of HL. (b) Average amplitudes of EPR spectra (no 3-(3,4-Dichlorophenyl)-1,1-dimethylurea [DCMU], closed squares; with DCMU, closed triangles). The significance level ($P < 0.05$) is given for the samples measured in the absence of DCMU. (c) Catalase activity of the crude extract. 100% corresponds to an activity of 10 μmol O_2 mg $protein^{-1}$ min^{-1} when 1 mM H_2O_2 was added as substrate. The significance level ($P < 0.05$) is given for the samples for time 0, 5 and 15–60 min. The samples between 1–4 and 7.5 min fall into the significance category bd ($n = 3$–13). (d) SOD (open circles) and ascorbate peroxidase activity (open triangles) of the crude extract. 100% corresponds to a superoxide dismutase (SOD) activity of 9.6 μmol mg $protein^{-1}$ min^{-1}. 100% corresponds to an APX activity of 10 μmol dehydroascorbate mg $protein^{-1}$ min^{-1} when 2 mM H_2O_2 was added as substrate. Error bars represent SD (three to six independent experiments).

No EPR signal was detected without Fe-EDTA, confirming that the measurement indeed reports H_2O_2 levels (data not shown). The detected H_2O_2 most likely originates from the disproportionation of $O_2^{\bullet-}$ generated by photosystem I during excess illumination. When the photosynthetic electron transfer chain was blocked using 3-(3,4-Dichlorophenyl)-1,1-dimethylurea, an inhibitor that binds to the Q_B-binding pocket of photosystem II, no increase in H_2O_2 was observed and its levels remained constant (Fig. 1b). Photorespiration as a source for H_2O_2 can be excluded since *C. reinhardtii* lacks glycolate oxidase activity (Goyal 2002). The amplitude of the EPR signal recorded was proportional to the amount of H_2O_2 present in the samples (Supporting Information Fig. S1; see also Mubarakshina *et al*. 2010). For our assay, the question arose if the spin trap was taken up by the cells and reacted with H_2O_2 inside the cells or whether the assay detected H_2O_2 secreted by the cells into the medium. H_2O_2 is known to be secreted by green algae into the medium (Zepp, Skurlatov & Pierce 1987). We tested the localization of the spin trap by centrifugation of the cells and re-suspension in fresh media. After centrifugation, the same signal size was obtained in the supernatant as in the sample before centrifugation. This indicates that most of the signal originates from the medium, but it does not exclude the possibility that the trap enters the cells. Upon addition of fresh medium to the collected cells followed by centrifugation, the signal size decreased proportionally with the dilution factor. This indicates that the trap diffused freely between the cells and the medium. In addition, the distribution of the spin trap between cells and medium was tested by adding the EPR-active spin adduct to dark-adapted cells so as to avoid variations in H_2O_2 production during the experiment. In this case, when hydroxyethyl-POBN was added to the cells, the distribution of the signal between the pellet and the supernatant was equal. In addition, after several dilution and centrifugation steps, a signal of a size proportional to the dilution factor was measured. This indicates that the spin trap diffuses freely between the cells and medium in the case of the cell-wall-less green alga *C. reinhardtii* D66 and that H_2O_2 is detected both in the medium and inside the cells.

When *C. reinhardtii* cells were shifted from dark to HL (700 μmol quanta m^{-2} s^{-1}), there was an immediate

accumulation of H_2O_2 that lasted for 10 min and then dropped slowly to a steady-state level (Fig. 1a,b). During the first 10 min of HL, the rate of detected H_2O_2 accumulation was approximately 30 μmol H_2O_2 mg Chl^{-1} h^{-1} (the signal size at $t = 0$ corresponds to 6 μmol H_2O_2; see Supporting Information Table S1). In response to HL, catalase activity decreased during the first 2 min of exposure recovering after 7 min to the maximum activity. The 2 min interval was consistent with the activation of Calvin–Benson cycle enzymes in *C. reinhardtii* (Farr, Huppe & Turpin 1994). Then, catalase activity decreased for the second time, reaching another minimum at about 10–15 min, corresponding to the maximum H_2O_2 level (Fig. 1c).

In addition to catalase, changes in other enzymes of the antioxidant system could contribute to the transient accumulation of H_2O_2. Therefore, we investigated changes in the activities of APX and SOD. In contrast to catalase activity, APX activity remained unchanged during HL exposure, while SOD activity slightly decreased at first and then increased after 20 min of HL (Fig. 1d) and stayed constant during the HL illumination.

Role of the catalase in the response of *C. reinhardtii* to HL stress

In order to demonstrate the importance of catalase in the response of *C. reinhardtii* to HL intensity, we generated catalase knock-down mutants using the amiRNA technology (Molnar *et al*. 2009). By transforming *C. reinhardtii* D66 cell-wall-less strain with the catalase amiRNA-expressing vector pChlamiRNA3int, we obtained several transformants exhibiting a significant decrease of catalase protein level (Fig. 2a). We noticed that the level of expression of catalase was mainly depending on growth phase (resting *Chlamydomonas* exhibiting lower levels of catalase compared to *Chlamydomonas* maintained in exponential phase state) and not on the transformant considered. We selected two types of transformants for further analysis: amiRNAcat40 and amiRNAcat15 exhibiting a residual catalase activity of 40 or 15%, respectively, compared to the parental strain.

Wild-type (WT) cells grew well on TAP plates supplemented with 0.8 mM H_2O_2, whereas amiRNAcat40 transformants barely grew on 0.8 mM H_2O_2 and amiRNAcat15 cells did not grow on medium supplemented with 0.8 mM and only slightly with 0.4 mM H_2O_2 (Fig. 2). In the dark, the amiRNAcat40 line accumulated 1.5-fold more H_2O_2, while amiRNAcat15 accumulated 1.6-fold less H_2O_2 than the WT strain (Supporting Information Table S1).

For both amiRNA lines, the time course of H_2O_2 accumulation was different from the WT strain, showing no transient

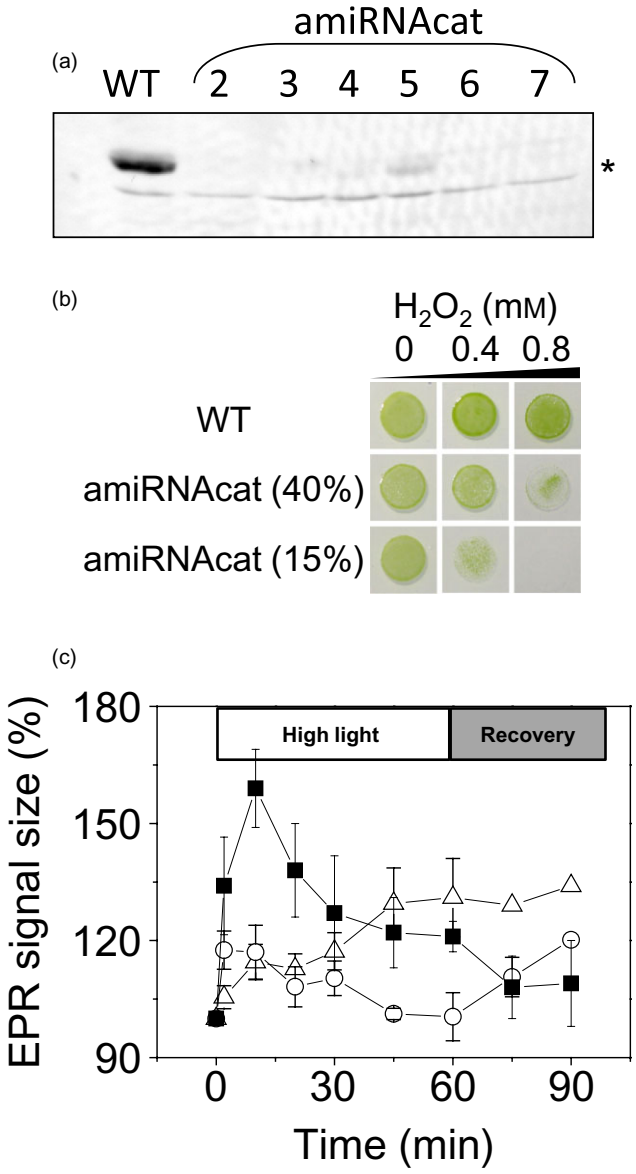

Figure 2. Sensitivity towards H_2O_2 and H_2O_2 production after a dark to high light shift in catalase knock-down *Chlamydomonas* mutants. (a) Catalase protein levels in wild-type (WT) and amiRNA mutant strains (2–7) analysed by Western blot using anti-catalase antibodies. The asterisk corresponds to a specific signal that can be used as a loading control. (b) Comparison of the growth of WT *Chlamydomonas* and catalase knock-down mutants (amiRNAcat) with a remaining catalase activity of 40 or 15% spotted on TAP plates supplemented with H_2O_2 at given concentrations. (c) Average amplitude of electron paramagnetic resonance (EPR) spectra obtained for catalase knock-down *Chlamydomonas* mutants with a remaining catalase activity of 40% (closed circles) and with a remaining catalase activity of 15% (open circles) collected at given time points after a dark to high light (700 μmol quanta m^{-2} s^{-1}) switch during 60 min, then collected during 30 additional minutes of recovery under normal illumination (70 μmol quanta m^{-2} s^{-1}). The values are given as a percentage, 100% corresponding to the value measured at time 0. Error bars represent SD (four independent experiments). Significant differences ($P < 0.05$) were observed between amiRNAcat (15%) and amiRNAcat (40%) at illumination times longer than 30 min. When the strains were analysed independently, significant differences were obtained for amiRNAcat (40%) at illumination times longer than 30 min, while no significant differences were obtained between the different time points for amiRNAcat (15%). For clarity, the data points for WT from Fig. 1b are shown.

Table 1. Ascorbate, total free-thiol content, ascorbate peroxidase (APX) and superoxide dismutase (SOD) activity of amiRNAcat15, amiRNAcat40 and wild type (WT) grown under continuous light (70 μmol m^{-2} s^{-1})

	Reduced ascorbate (nmol mg chl^{-1})	Total ascorbate (nmol mg chl^{-1})	Total free-thiol content (μmol mg ch^{-1})	APX activity (μmol min^{-1} mg chl^{-1})	SOD activity (μmol min^{-1} mg chl^{-1})
amiRNAcat15	147 ± 24	168 ± 30	4.1 ± 1.3[c]	4.8 ± 0.5[b]	11 ± 3[b]
amiRNAcat40	202 ± 67	241 ± 83	8.5 ± 0.7[b]	7.5 ± 2.1[ab]	33 ± 11[b]
WT	156 ± 57	175 ± 62	11.4 ± 2.0[a]	10.4 ± 1.9[a]	61 ± 20[a]

Error bars represent SD (three to six independent experiments). Letters indicate the different significance categories. The significance level ($P < 0.05$) is given for total free-thiol content, APX and SOD activity.

accumulation. Instead, the amiRNA lines showed an almost linear increase in H_2O_2 levels in the case of amiRNAcat40 or, in the case of amiRNAcat15, a slight increase for the first 2 min followed by a constant decrease in H_2O_2 during HL (Fig. 2c). Photosynthetic activity decreased considerably in both amiRNA lines, and the transformants with 15% catalase activity were more severely affected than those with 40% catalase activity (Supporting Information Fig. S2). The free-thiol content, most likely glutathione, was significantly decreased in these mutant strains, while the ascorbate content remained unchanged. Furthermore, APX and SOD activity were reduced compared to the parental strain (Table 1). These results show that the capacity of stress defence in strains with strongly reduced catalase activity was already lower and that these strains were not able to cope with sudden exposure to HL.

Changes in gene expression levels in response to HL exposure

To show whether a transient accumulation of H_2O_2 was coordinated to changes in the expression levels of typical oxidative and general stress response genes, we analysed the expression of the ascorbate peroxidase gene *APX*, the glutathione peroxidase homologous gene *GPX5/GPXH*, the glutathione-S-transferase *GSTS1* and three genes of the heat shock proteins *HSP22C*, *HSP70A* and *HSP70B* in the WT parental strain and amiRNAcat15 (Fig. 3). Even though most of these genes are known to respond to multiple signals (Fischer *et al*. 2005), the expression of *HSP22C* and *HSP70A* genes followed the accumulation of H_2O_2 in the WT strain, with a transient induction peaking after 30 min and a decrease to lower levels of expression during the next 30 min of exposure to HL. The expression level of HSP22C was strongly induced after 30 min of HL in the WT, while only a small increase in the expression level was observed in amiRNAcat15. The expression levels of *HSP70A* were very similar to WT and amiRNAcat15. Furthermore, the expression of *HSP70B* was increased to its maximum within 30 min for both, WT and amiRNA15; however, the expression level stayed high during HL exposure. The expression of *APX* and *GPXH*, on the other hand, increased continuously during the 60 min of HL exposure before coming back to their initial levels when the cells were shifted to 70 μmol $quantam^{-2}$ s^{-1} (recovery period). The same trend was observed in the mutant strain; however, the expression level of *APX* and *GPXH* at 60 min was significantly higher in WT compared to amiRNAcat15. *GSTS1* expression remained unaffected by HL in the WT strain. In the amiRNAcat15 mutant, expression of the *HSP22C* and *GSTS1* genes was already higher than in WT during the dark period and only slightly increased or even decreased after the HL shift to reach similar levels compared to the WT strain after 60 min of exposure (Fig. 3).

Regulation of catalase activity

Catalase has been identified as a target of reduced thioredoxins and its activity may be regulated via the reduction state of cysteine residues as it is inactivated by reduced thioredoxin and by DTT (Lemaire *et al*. 2004). Low DTT concentrations inhibited the catalase activity not only of the purified recombinant *C. reinhardtii* enzyme but also of the crude extracts from *C. reinhardtii* cells (Fig. 4a). A similar inhibitory effect was observed in *Arabidopsis thaliana* leaf extracts, while, in contrast, bovine catalase was almost unaffected. However, at DTT concentrations higher than 0.5 mM, activity loss occurred in all tested catalases, suggesting a general sensitivity of catalase to strongly reducing conditions. In order to determine if DTT treatment could lead to differences in organization of the protein, we analysed recombinant *Chlamydomonas* catalase in native gels (Fig. 4b). In-gel staining of catalase activity revealed two bands in the absence of DTT, the lower band being much more intense, while after DTT treatment, the lower band almost completely disappeared, confirming the loss in activity. In contrast, bovine catalase remained unaffected by the DTT treatment. A putative conformational change of catalase upon reduction was further investigated by following the dependence of tryptophan fluorescence on the redox potential of the buffer (Fig. 4c). The resulting sigmoid curve suggested that the changes in the protein conformation took place with a midpoint potential of –330 mV at pH 8.0, which is compatible with redox regulation of catalase by thioredoxins. However, the redox profile could not be fitted to a two-electron process but to a one-electron process, which eliminated changes in structure due to oxido-reduction of disulfide bridges. Concomitant to fluorescence changes, catalase activity decreased when the redox potential was lowered and was completely inhibited at –340 mV.

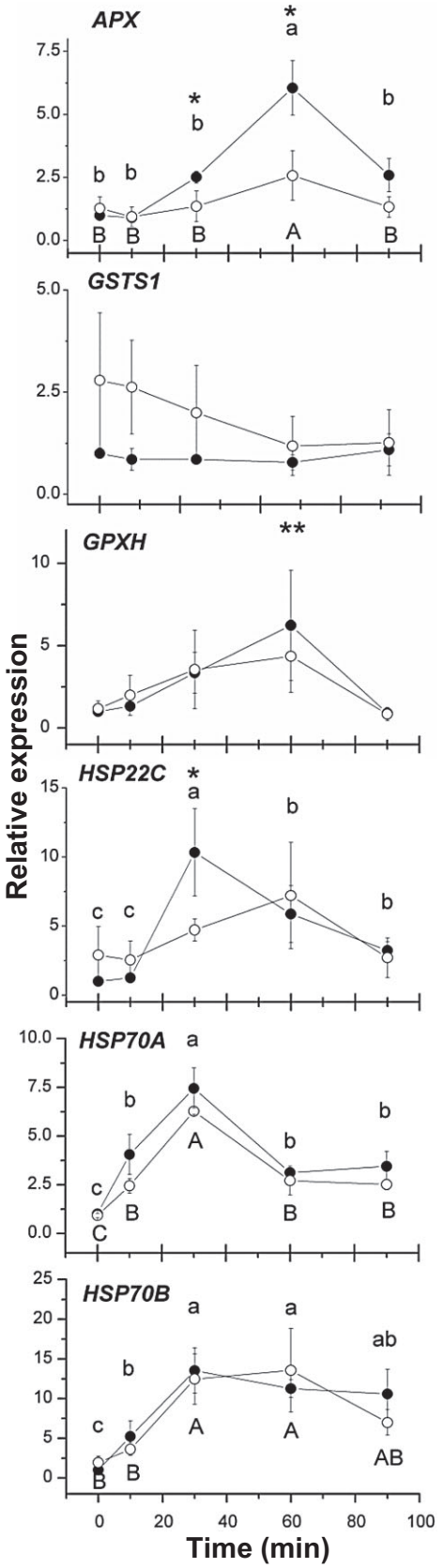

Figure 3. Expression of stress-related genes after a dark to high light (HL) shift in *Chlamydomonas reinhardtii*. Expression levels of a glutathione peroxidase (*GPXH*), a glutathione-S-transferase (*GSTS1*) and three genes coding for heat shock proteins (*HSP22C, HSP70A* and *HSP70B*) have been determined in the wild-type (WT) (closed symbols, full lines) and amiRNAcat15 mutant (open symbols, dashed lines) after a dark to HL shift by Q-PCR. Cultures were transferred from the dark to HL (700 μmol quanta m^{-2} s^{-1}) for 60 min and then back to 70 μmol quanta m^{-2} s^{-1} for 30 min (recovery period from 60 to 90 min). Relative expression compared to the WT strain at time zero of the HL shift was calculated and average expression from three independent experiments with standard error bars are shown. The significance level ($P < 0.05$) is given for the samples at the different time points (small letters: WT, capital letters: mutant, stars: comparison of WT and mutant at the same time point).

DISCUSSION

Catalase activity seems to play a major role in H_2O_2 detoxification in *C. reinhardtii*. As shown in Fig. 1, the peak of H_2O_2 during the first 10 min of HL correlated with a reversible loss of catalase activity in the WT strain. This suggests that high catalase activity keeps H_2O_2 levels low in the dark, but that a decrease of its activity allows a transient increase after the HL shift. In the amiRNAcat15 and cat40 mutants, this H_2O_2 peak was no longer observed, supporting the role of catalase in this transient accumulation of H_2O_2. In higher plants, it is thought that most of the H_2O_2 generated inside the chloroplast is detoxified by APX, while in green algae the amount and distribution of APX might be highly variable depending on the species and growth conditions (Ishikawa & Shigeoka 2008). For example, it has been shown recently that APX activity is 17-fold lower in *C. reinhardtii* compared to the marine *Chlamydomonas* strain W80 (Tanaka *et al.* 2011). However, SOD activity is doubled and catalase activity is even tripled in *C. reinhardtii* compared to *Chlamydomonas* sp. W80 (Tanaka *et al.* 2011). In the present study, SOD activity increased after 20 min of HL exposure and stayed constant during the illumination time (Fig. 1). It has been reported previously that transcript level of Fe-SOD increased significantly after 2 h or more of HL exposure of *C. reinhardtii* (McKim & Durnfod 2006). Since changes in SOD activity did not correlate with the increase in the H_2O_2 level during the first 10 min of HL exposure, we conclude that this enzyme activity is not rate limiting for the fast accumulation of H_2O_2 after HL shift in our experiments.

In vitro, DTT treatment inactivated *Chlamydomonas* catalase (Fig. 4a) (Lemaire *et al.* 2004; Shao *et al.* 2008). The inactivation of the enzyme may be related to a change in the molecular organization of the protein. Active catalase in *C. reinhardtii* is a dimer (Kato *et al.* 1997), while it is a tetramer in higher plant (Mhamdi *et al.* 2010). While higher plants contain three catalase genes, only one catalase gene (CAT1, A8J537, UNIPROT) is present in the *Chlamydomonas* genome. In higher plants, catalases are localized to peroxisomes, while in *C. reinhardtii*, which lacks leaf-like peroxisomes, the enzyme is most likely located in cytosolic microbodies that were visualized by sub-cellular

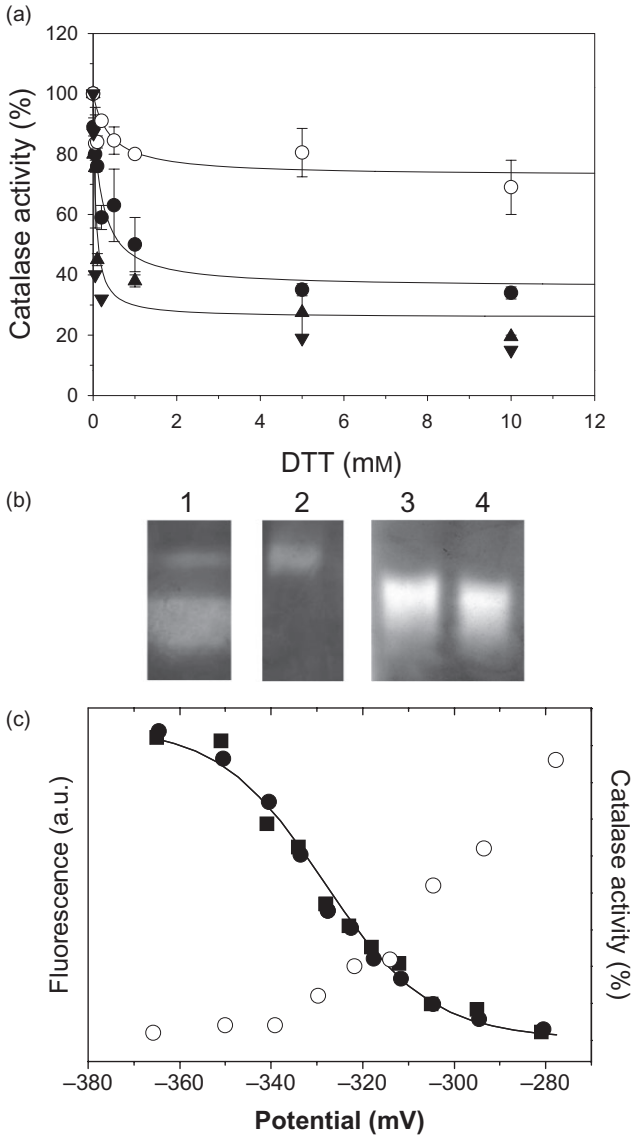

Figure 4. Characterization of *Chlamydomonas* catalase in respect to its sensitivity to dithiothreitol (DTT). (a) Catalase inactivation upon treatment with DTT. Catalase activity from bovine catalase (open circles), recombinant *Chlamydomonas reinhardtii* catalase (filled up triangles), from total soluble protein extracts of *C. reinhardtii* (closed down triangles) or *Arabidopsis thaliana* (filled circles), respectively, after 5 min of incubation at 20 °C with given concentrations of DTT. Error bars represent SD (three to six independent experiments). (b) In-gel activity staining of catalase activity in *C. reinhardtii* crude extracts (lanes 1, 2) or bovine catalase (lanes 3,4) following separation on 8% native gel after 15 min of incubation at 20 °C without (lanes 1,3) or with (lanes 2,4) 50 mM DTT. (c) Dependency of tryptophan fluorescence (two experiments on independent catalase preparations shown as closed squares and closed circles) and of the activity (open circles) of recombinant *C. reinhardtii* catalase on the redox potential of the medium. Samples were incubated for 90 min at different redox potentials imposed by various ratios of dithiothreitol/trans-4,5-dihydroxy-1,2-dithiane at pH 8.0.

localization of green fluorescent proteins fused to several putative *Chlamydomonas* peroxisomal targeting sequences (Hayashi & Shinozaki 2012). Figure 4 shows that inactivation of catalase activity may be related to a conformational change. Moreover, the redox dependence of the tryptophan fluorescence suggests that the reduction of a single cysteine residue may be responsible for the inactivation of the enzyme. Although redox titrations exclude the possibility that the activity is regulated via oxido-reduction of a disulfide bridge, this does not rule out regulation by TRXs, which can also reduce other forms of oxidized cysteines such as sulfenates or nitrosothiols (Benhar, Forrester & Stamler 2009; Tarrago *et al*. 2010). A sequence comparison of the catalases CAT1, CAT2 and CAT3 from *A. thaliana* and the catalase from *C. reinhardtii* reveals that Cys_{230} is conserved among these species. Homology modelling using Swiss model (Arnold *et al*. 2006) of the structure of *C. reinhardtii* catalase based on the 2.3 Å structure of bovine catalase (Ko *et al*. 1999) shows that Cys_{230} is located at the surface. Redox modification of this cysteine residue, under the control of TRX, may therefore play a role in the regulation of catalase conformation in response to plastid-derived redox signals and account for the changes observed in native gels (Fig. 4b). Other potential mechanisms may also contribute to the regulation of catalase activity, such as salicylic acid, NO, diurnal rhythms and Ca^{2+}/calmodulin as previously described in higher plants (Mhamdi *et al*. 2010). Furthermore, catalase is a light-sensitive enzyme. However, the $t_{1/2}$ of catalase in rye was shown to be 3–4 h when the leaves were illuminated with 520 μmol quanta m^{-2} s^{-1} (Hertwig, Streb & Feierabend 1992), while the changes in catalase activity observed here occurred within minutes.

In the *C. reinhardtii* WT strain, three HSP genes known to be induced by H_2O_2 (Fischer *et al*. 2005; Shao *et al*. 2007) responded to the dark-HL shift with kinetics following the H_2O_2 accumulation (Fig. 3). In the amiRNAcat15 mutant, one of them, the *HSP22C* gene, showed already higher expression in the dark and slower induction after the dark-HL shift compared to the WT strain. This indicates that the expression of this gene is mostly regulated by H_2O_2. The *APX* gene and, also to a lower extent, the *GPXH* gene showed higher expression levels in WT than in amiRNAcat15 after prolonged exposure to HL. These genes also seem to be induced by increased H_2O_2 levels, whereas the expression of *HSP70A* and *HSP70B* might mainly respond to other signals during the dark-HL shift (Shao *et al*. 2007). Similarly, other genes like *GPXH* or *GSTS1* have been shown to respond more specifically to other signals like singlet oxygen or reactive electrophile species, respectively, and thus exhibited different induction kinetics after dark-HL treatment (Fig. 3) (Fischer *et al*. 2006, 2012). Still, an increase in *GSTS1* expression level in the amiRNAcat15 mutant indicates that catalase activity is essential to control H_2O_2 levels in the dark and to prevent oxidative stress and cellular damage in the cell. However, after shifting to HL conditions, other defence mechanisms might be required like the chloroplast-directed *HSP22C* and *HSP70B* proteins, which are probably involved in the protection of the photosynthetic apparatus from photodamage (Schroda *et al*. 1999). Thus, H_2O_2 might function as specific plastid signal to induce

the expression of chloroplast-localized proteins after the dark to light transition to protect the photosystems.

In conclusion, the data presented provide support for the following hypothesis: upon exposure of *C. reinhardtii* to HL, overreduction of the photosynthetic electron transfer chain and downstream acceptors, such as $NADP^+$ and TRXs, and a production of H_2O_2, which diffuses out of the chloroplast. Meanwhile, a decrease in catalase activity that is linked to the reduction state of the photosynthetic electron transport chain allows H_2O_2 concentrations to increase. This H_2O_2 accumulation induces a signalling event that is transmitted to the nucleus and modulates the expression of protection enzymes. In HL, down-regulation mechanisms of the electron transport chain take place (such as protein phosphorylation and degradation of the D1 protein; see Edelman & Mattoo 2008; Rochaix 2011). Consequently, the reduction pressure and the reduction state of acceptors are lowered so that catalase is reactivated. Thus, H_2O_2 concentration is lowered, thereby avoiding oxidative cellular damages and switching off the H_2O_2 signal.

This signalling mechanism shares strong similarities with the floodgate theory proposed for H_2O_2 signalling in mammals where down-regulation of 2-Cys Prx would allow a transient accumulation of H_2O_2 required for signalling (Wood *et al*. 2003; Woo *et al*. 2010). In plants, the importance of 2-Cys Prx in signalling remains unclear and the lack of a specific assay to measure 2-Cys Prx activity in total extracts precluded their analysis in our system. However, the roles of 2-Cys Prx are likely different in plants compared to mammals. These enzymes are only present in chloroplasts and are required for protection of the photosynthetic machinery (Baier & Dietz 1999). Moreover, APX and CAT play a more prominent role in plants (Mhamdi *et al*. 2010; Foyer & Noctor 2011). Therefore, although plant 2-Cys Prx may play some role in signalling (Dietz 2011), the present study points towards an important role of catalase in a floodgate model of H_2O_2 signalling in green algae.

ACKNOWLEDGMENTS

We would like to thank S. Un and D. Kirilovsky (both from CEA Saclay) for fruitful discussions and critical reading of the manuscript. This work was supported by the Agence Nationale de Recherche ANR-09-BLAN-0005-01. T.R. was supported by EU FP7 Marie Curie Initial Training Network HARVEST (FP7 Project No. 238017).

REFERENCES

Apel K. & Hirt H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* **55,** 373–399.

Arnold K., Bordoli L., Kopp J. & Schwede T. (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22,** 195–201.

Baier M. & Dietz K.J. (1999) Protective function of chloroplast 2-cysteine peroxiredoxin in photosynthesis. Evidence from transgenic *Arabidopsis*. *Plant Physiology* **119,** 1407–1414.

Bechtold U., Richard O., Zamboni A., Gapper C., Geisler M., Pogson B., Karpinski S. & Mullineaux P.M. (2008) Impact of chloroplastic- and extracellular-sourced ROS on high light-responsive gene expression in *Arabidopsis*. *Journal of Experimental Botany* **59,** 121–133.

Benhar M., Forrester M.T. & Stamler J.S. (2009) Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nature Reviews Molecular Cell Biology* **10,** 721–732.

Biteau B., Labarre J. & Toledano M.B. (2003) ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* **425,** 980–984.

op den Camp R.G., Przybyla D., Ochsenbein C., *et al*. (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in *Arabidopsis*. *The Plant Cell* **15,** 2320–2332.

Dietz K.J. (2011) Peroxiredoxins in plants and cyanobacteria. *Antioxidants & Redox Signaling* **15,** 1129–1159.

Edelman M. & Mattoo A.K. (2008) D1-protein dynamics in photosystem II: the lingering enigma. *Photosynthesis Research* **98,** 609–620.

Farr T.J., Huppe H.C. & Turpin D.H. (1994) Coordination of chloroplastic metabolism in N-limited *Chlamydomonas reinhardtii* by redox modulation (I. The activation of phosphoribulosekinase and glucose-6-phosphate dehydrogenase is relative to the photosynthetic supply of electrons). *Plant Physiology* **105,** 1037–1042.

Fischer B.B., Krieger-Liszkay A. & Eggen R.I.L. (2005) Oxidative stress induced by the photosensitizers neutral red (type I) or rose bengal (type II) in the light causes different molecular responses in *Chlamydomonas reinhardtii*. *Plant Science* **168,** 747–759.

Fischer B.B., Eggen R.I., Trebst A. & Krieger-Liszkay A. (2006) The glutathione peroxidase homologous gene Gpxh in *Chlamydomonas reinhardtii* is upregulated by singlet oxygen produced in photosystem II. *Planta* **223,** 583–590.

Fischer B.B., Ledford H.K., Wakao S., Huang S.G., Casero D., Pellegrini M., Merchant S.S., Koller A., Eggen R.I.L. & Niyogi K.K. (2012) SINGLET OXYGEN RESISTANT 1 links reactive electrophile signaling to singlet oxygen acclimation in *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America* **109,** E1302–E1311.

Foyer C.H. & Noctor G. (2011) Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology* **155,** 2–18.

Gadjev I., Vanderauwera S., Gechev T.S., Laloi C., Minkov I.N., Shulaev V., Apel K., Inze D., Mittler R. & Van Breusegem F. (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in *Arabidopsis*. *Plant Physiology* **141,** 436–445.

Gough D.R. & Cotter T.G. (2011) Hydrogen peroxide: a Jekyll and Hyde signalling molecule. *Cell Death & Disease* **2,** e213.

Goyal A. (2002) Glycolate metabolism in algal chloroplasts: inhibition by salicylhydroxamic acid (SHAM). *Physiologia Plantarum* **116,** 264–270.

Hayashi Y. & Shinozaki A. (2012) Visualization of microbodies in *Chlamydomonas reinhardtii*. *Journal of Plant Research* **125,** 579–586.

Hertwig B., Streb P. & Feierabend J. (1992) Light dependence of catalase synthesis and degradation in leaves and the influence of interfering stress conditions. *Plant Physiology* **100,** 1547–1553.

Ishikawa T. & Shigeoka S. (2008) Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms. *Bioscience, Biotechnology, and Biochemistry* **72,** 1143–1154.

Kampfenkel K., Van Montagu M. & Inze D. (1995) Extraction and determination of ascorbate and dehydroascorbate from plant tissue. *Analytical Biochemistry* **225,** 165–167.

Kato J., Yamahara T., Tanaka K., Takio S. & Satoh T. (1997) Characterization of catalase from green algae *Chlamydomonas reinhardtii*. *Journal of Plant Physiology* **151,** 262–268.

Ko T.P., Day J., Malkin A.J. & McPherson A. (1999) Structure of orthorhombic crystals of beef liver catalase. *Acta Crystallographica Section D: Biological Crystallography* **55,** 1383–1394.

Laloi C., Stachowiak M., Pers-Kamczyc E., Warzych E., Murgia I. & Apel K. (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 672–677.

Lemaire S.D., Guillon B., Le Maréchal P., Keryer E., Miginiac-Maslow M. & Decottignies P. (2004) New thioredoxin targets in the unicellular photosynthetic eukaryote *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 7475–7480.

McKim S.M. & Durnford D.G. (2006) Translational regulation of light-harvesting complex expression during photoacclimation to high-light in *Chlamydomonas reinhardtii*. *Plant Physiology and Biochemistry* **44,** 857–865.

Mene-Saffrane L., Jones A.D. & DellaPenna D. (2010) Plastochromanol-8 and tocopherols are essential lipid-soluble antioxidants during seed desiccation and quiescence in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 17815–17820.

Mhamdi A., Queval G., Chaouch S., Vanderauwera S., Van Breusegem F. & Noctor G. (2010) Catalase function in plants: a focus on *Arabidopsis* mutants as stress-mimic models. *Journal of Experimental Botany* **61,** 4197–4220.

Mittler R., Vanderauwera S., Suzuki N., Miller G., Tognetti V.B., Vandepoele K., Gollery M., Shulaev V. & Van Breusegem F. (2011) ROS signaling: the new wave? *Trends in Plant Science* **16,** 300–309.

Molnar A., Bassett A., Thuenemann E., Schwach F., Karkare S., Ossowski S., Weigel D. & Baulcombe D. (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *The Plant Journal* **58,** 165–174.

Mubarakshina M.M., Ivanov B.N., Naydov I.A., Hillier W., Badger M.R. & Krieger-Liszkay A. (2010) Production and diffusion of chloroplastic H_2O_2 and its implication to signalling. *Journal of Experimental Botany* **61,** 3577–3587.

Noctor G., Veljovic-Jovanovic S. & Foyer C.H. (2000) Peroxide processing in photosynthesis: antioxidant coupling and redox signalling. *Philosophical Transactions of the Royal Society B: Biological Sciences* **355,** 1465–1475.

Queval G., Neukermans J., Vanderauwera S., Van Breusegem F. & Noctor G. (2012) Day length is a key regulator of transcriptomic responses to both CO_2 and H_2O_2 in *Arabidopsis*. *Plant, Cell & Environment* **35,** 374–387.

Rahman I., Kode A. & Biswas S.K. (2006) Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols* **1,** 3159–3165.

Rochaix J.D. (2011) Regulation of photosynthetic electron transport. *Biochimica et Biophysica Acta* **1807,** 375–383.

Schroda M., Vallon O., Wollman F.A. & Beck C.F. (1999) A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. *The Plant Cell* **11,** 1165–1178.

Shao N., Krieger-Liszkay A., Schroda M. & Beck C.F. (2007) A reporter system for the individual detection of hydrogen peroxide and singlet oxygen: its use for the assay of reactive oxygen species produced *in vivo*. *The Plant Journal* **50,** 475–487.

Shao N., Beck C.F., Lemaire S.D. & Krieger-Liszkay A. (2008) Photosynthetic electron flow affects H_2O_2 signaling by inactivation of catalase in *Chlamydomonas reinhardtii*. *Planta* **228,** 1055–1066.

Stone J.R. & Yang S. (2006) Hydrogen peroxide: a signaling messenger. *Antioxidants & Redox Signaling* **8,** 243–270.

Tanaka S., Ikeda K., Miyasaka H., Shioi Y., Suzuki Y., Tamoi M., Takeda T., Shigeoka S., Harada K. & Hirata K. (2011) Comparison of three *Chlamydomonas* strains which show distinctive oxidative stress tolerance. *Journal of Bioscience and Bioengineering* **112,** 462–468.

Tarrago L., Laugier E., Zaffagnini M., Marchand C.H., Le Marechal P., Lemaire S.D. & Rey P. (2010) Plant thioredoxin CDSP32 regenerates 1-cys methionine sulfoxide reductase B activity through the direct reduction of sulfenic acid. *The Journal of Biological Chemistry* **285,** 14964–14972.

Woo H.A., Chae H.Z., Hwang S.C., Yang K.S., Kang S.W., Kim K. & Rhee S.G. (2003) Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* **300,** 653–656.

Woo H.A., Yim S.H., Shin D.H., Kang D., Yu D.Y. & Rhee S.G. (2010) Inactivation of peroxiredoxin I by phosphorylation allows localized H_2O_2 accumulation for cell signaling. *Cell* **140,** 517–528.

Wood Z.A., Poole L.B. & Karplus P.A. (2003) Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* **300,** 650–653.

Zepp R.G., Skurlatov Y.I. & Pierce J.T. (1987) Algal-induced decay and formation of hydrogen peroxide in water: its possible role in oxidation of anilines by algae. In *Photochemistry of Environmental Aquatic Systems* (eds W.J. Cooper & R.G. Zika), pp. 215–224. American Chemical Society, Washington, USA.

Received 18 July 2012; received in revised form 3 December 2012; accepted for publication 4 December 2012

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Calibration curve of the EPR signal using an H_2O_2 solution. Dependence of the EPR signals of H_2O_2-derived hydroxyl radicals on H_2O_2 concentration. A given concentration of H_2O_2 was added to TAP medium and incubated for 5 min with 50 mM 4-POBN, 4% ethanol, 50 μM Fe-EDTA.

Figure S2. Photosynthesis after a dark to high light switch in wild-type *C. reinhardtii* and in catalase knock-down mutants. Photosynthesis was measured in the presence (circles) or absence (diamonds) of 1 mM $NaHCO_3$ in wild-type and catalase knock-down mutants exhibiting 40 or 15% residual catalase activity following a dark to high light (700 μmol quanta m^{-2} s^{-1}) shift, and during an additional 30 min recovery at 70 μmol quanta m^{-2} s^{-1}. Typical O_2 evolution curves are shown, representative of at least three independent experiments. Measurements of photosynthesis were performed in a liquid-phase oxygen electrode chamber (Hansatech Instruments, Norfolk, England). Photosynthetic activity was measured in TAP medium under saturating white light.

Table S1. H_2O_2 concentration in the cultures as determined by the spin-trapping assay. Chlorophyll concentration of the culture: 10 μg mL^{-1}. Results are expressed as mean bars represent ± SD (3–6 independent experiments).