The glutathione peroxidase homologous gene from *Chlamydomonas reinhardtii* is transcriptionally up-regulated by singlet oxygen

Urs Leisinger, Karin Rüfenacht, Beat Fischer, Manuel Pesaro, Arik Spengler, Alexander J. B. Zehnder and Rik I. L. Eggen*

Department of Environmental Microbiology and Molecular Ecotoxicology, Swiss Federal Institute for Environmental Science and Technology (EAWAG), Ueberlandstrasse 133, P.O. Box 611, 8600 Duebendorf, Switzerland (*author for correspondence; e-mail eggen@eawag.ch)

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Abstract

The glutathione peroxidase homologous gene (*Gpxh* gene) in *Chlamydomonas reinhardtii* is up-regulated under oxidative stress conditions. The *Gpxh* gene showed a remarkably strong and fast induction by the singlet oxygen-generating photosensitizers neutral red, methylene blue and rose Bengal. The *Gpxh* mRNA levels strongly increased, albeit much more slowly, upon exposure to the organic hydroperoxides *tert*-butyl hydroperoxide (t-BOOH) and cumene hydroperoxide. In contrast, the *Gpxh* mRNA levels were only weakly induced by exposure to the superoxide-generating compound paraquat and by hydrogen peroxide. A comparison of the *Gpxh* mRNA levels with those of the heat shock protein HSP70A and the iron superoxide dismutase gene showed qualitative and quantitative differences for the three genes under oxidative stress conditions tested. The *Gpxh* gene is specifically induced by singlet-oxygen photosensitizers and the relative induction by other compounds is much weaker for *Gpxh* than for the other genes investigated. Using *Gpxh* promoter fusions with the arylsulfatase reporter gene, we have shown that the *Gpxh* was transcriptionally up-regulated by singlet-oxygen photosensitizers. It is also shown that the *Gpxh* promoter contains a region between 104 and 179 bp upstream of the transcription start that is responsible for the mRNA up-regulation upon exposure to $^1\text{O}_2$ but not t-BOOH. Within this region a regulatory sequence homologous to the mammalian cAMP response element (CRE) and activator protein 1 (AP-1) binding site was identified within a 16 bp palindrome.

Abbreviations: $^1\text{O}_2$, singlet oxygen; CU-OOH, cumene hydroperoxide; *Fesod*, iron-dependent superoxide dismutase gene; *Gpxh*, glutathione peroxidase homologous; *Hsp70a*, heat shock protein 70A; MB, methylene blue; NR, neutral red; RB, rose Bengal; TAP, Tris-acetate-phosphate; t-BOOH, *tert*-butyl hydroperoxide.

Introduction

Molecular oxygen is essential for many organisms, and at the same time causes damage to cells. Although molecular oxygen is not very reactive, it can be activated to the reactive oxygen species (ROS) superoxide

radical anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{OH}^{\cdot}$) by partial reduction. In addition, molecular oxygen can be activated to the ROS singlet oxygen ($^1\text{O}_2$) either by transfer of excitation energy or in redox reactions (Halliwell and Gutteridge, 1989; Elstner, 1991; Bensasson *et al.*, 1993). ROS are produced during normal metabolic activities and their generation augments upon exposure to strong illumination, UV irradiation, pollutants or pathogens.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF014927.

Organisms possess different mechanisms to sense and scavenge ROS, or to repair ROS-caused damage (Halliwell and Gutteridge, 1989; Takeda *et al.*, 1997). H_2O_2 - and $\text{O}_2^{\cdot-}$ -induced responses have been studied in great detail in both prokaryotic and eukaryotic organisms. However, knowledge on singlet-oxygen ($^1\text{O}_2$)-induced responses is rather restricted despite its potential importance for cells (Briviba *et al.*, 1997; Ryter and Tyrrell, 1998). Many cellular molecules can act as photosensitizers and thus give rise to $^1\text{O}_2$ (Halliwell and Gutteridge, 1989; Neverov *et al.*, 1996; Girotti, 1998; Ryter and Tyrrell, 1998). In addition, $^1\text{O}_2$ can be formed inside cells by redox reactions (Duran, 1982; Siesow, 1991; Khan and Khasa, 1994; Briviba *et al.*, 1997; Di Mascio *et al.*, 1997; Cadenas and Sies, 1998; Ryter and Tyrrell, 1998). Since photosynthesis is a major site of $^1\text{O}_2$ generation, photosynthetically active cells are particularly endangered. This occurs especially during acceptor side photoinhibition when the plastoquinone pool is fully reduced, and, in *C. reinhardtii*, during water splitting (Evans and Nugent, 1993; Macpherson *et al.*, 1993; Telfer *et al.*, 1994; Hideg *et al.*, 1994a, b, 1998; Van Mieghem *et al.*, 1995; Roudyk *et al.*, 1996; Kalai *et al.*, 1998). In view of these many pathways of $^1\text{O}_2$ generation and of the high reactivity of $^1\text{O}_2$, it seemed probable that photosynthetic organisms also possess specific adaptations to $^1\text{O}_2$ at the level of gene expression.

Glutathione peroxidases (GPX) are considered as key enzymes in the anti-ROS defence in animals, therefore giving rise to an extensive study of both enzymes and the corresponding genes (Ursini *et al.*, 1995). *Gpx*-homologous (*gpxh*) sequences have been isolated from different plant species (Criqui *et al.*, 1992; Holland *et al.*, 1993; Sugimoto *et al.*, 1997; Depege *et al.*, 1998; Roeckel-Drevet *et al.*, 1998) and from the single-cellular green alga *Chlamydomonas reinhardtii* (Leisinger *et al.*, 1999). The proteins encoded by these genes belong to the family of selenium-independent GPX-like proteins (Eshdat *et al.*, 1997). It has been shown that the expression of these genes is induced upon exposure to oxidative stress, as well as to other biotic and abiotic stresses. These stresses include exposure to paraquat (methyl viologen), *tert*-butylhydroperoxide (t-BOOH), H_2O_2 , HgCl_2 , NaCl, mechanical stimulation, wounding and infections (Criqui *et al.*, 1992; Holland *et al.*, 1993; Gueta-Dahan *et al.*, 1997; Depege *et al.*, 1998; Roeckel-Drevet *et al.*, 1998; Leisinger *et al.*, 1999). Furthermore, the expression of *gpxh* genes has been found to be tissue-specific in higher plants (Depege *et al.*, 1998;

Roeckel-Drevet *et al.*, 1998). It has been speculated that their function in the oxidative stress defence might be the reduction of organic hydroperoxides that are not accessible to other detoxifying enzymes. Alternatively, *gpxh* might encode an enzyme whose activity is required under overexposure to ROS when the other antioxidative defence mechanisms are not able to inactivate all the ROS present (Beeor-Tzahar *et al.*, 1995; Tang *et al.*, 1995). Regulation of *gpxh* expression in the various organisms is poorly understood.

We present mRNA profiles of the *Gpxh* from *C. reinhardtii* exposed to various compounds and conditions that generate different types of oxidative stress. *C. reinhardtii* has been established as a model organism to study complex biological processes (Rochaix, 1995; Rochaix *et al.*, 1998; Mendez-Alvarez *et al.*, 1999) and was used in this study to determine the function of *gpxh* genes in photosynthetic organisms as well as its regulated expression. Interestingly, the *Gpxh* gene is very fast, specific and strongly up-regulated transcriptionally by $^1\text{O}_2$. To our knowledge, the *C. reinhardtii* *Gpxh* gene is the first gene in photosynthetic organisms which is known to be $^1\text{O}_2$ -induced.

Materials and methods

Strains and culture conditions

C. reinhardtii strains were generously provided by E. Harris. Strain cw_{15arg7mt}⁻ (CC-1618) and wild type (CC-125) (Harris, 1989) were cultured in Tris-acetate-phosphate medium (TAP) (Harris, 1989). For the induction experiments, not more than 350 ml of culture were grown in 2-litre Erlenmeyer flasks in order to avoid CO₂ limitation. The cultures were agitated on rotatory shakers (150 rpm) under constant illumination at 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR, 400–700 nm) at 25 °C (standard conditions). Cells were grown on solidified TAP containing 1% agar under a cycle of 14 h illumination (25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) and 10 h darkness at 25 °C. Chlorophyll-deficient, protoporphyrin IX-accumulating mutant strains (CC-52, CC-339 and CC-2398) were grown under the same conditions in the dark. When required, media were supplemented with 50 mg/l arginine and 50 mg/l ampicillin. For mRNA induction experiments, several cultures of *C. reinhardtii* cw_{15arg7mt}⁻ were grown to a density of ca. 5×10^6 cells per ml in TAP, mixed together, and divided into 120 ml subcultures in 500 ml Erlenmeyer

Figure 1. Expression of *Gpxh*, *Hsp70a*, *Fesod* under oxidative stress conditions. Total RNA was extracted from cultures of *C. reinhardtii* cw₁₅arg7 before exposure to chemicals and 30 min and 3 h after addition of H₂O₂, paraquat, t-BOOH and NR at the concentrations indicated. From each culture, 20 μ g of total RNA extracted 30 min and 3 h after exposure to stress were loaded and hybridized with *Gpxh* as a probe. From cultures that died during the run of the experiment, only the RNA extracted 30 min after exposure was blotted.

Figure 2. Time course of *Gpxh* expression upon exposure to 5 μ M NR and 60 μ M CU-OOH in the light. Northern blot analysis of RNA extracted before the addition of NR and CU-OOH and 7, 14, 20, 40 and 80 min after addition.

flasks that had been exposed to the different stress conditions. Total RNA was extracted from 40 ml aliquots taken at regular intervals (see Results).

Stock solutions of the chemicals were made in water, except for nitrofen (Promochem) which was dissolved in DMSO. The addition of DMSO alone did not alter mRNA levels (data not shown).

E. coli DH5 α (Sambrook *et al.*, 1989) was used for routine cloning experiments, and the *dam-3 E. coli* strain GM119 (Boyer and Roulland-Dussoix, 1969) was used to obtain pUC28 with an unmethylated *Clal* restriction site. The strains were grown on LB at 37 $^{\circ}$ C.

Nucleic acids

Full-length cDNA of *Gpxh* from plasmid pGPX β 9 was used for the detection of *Gpxh* (Leisinger *et al.*, 1999). A 3.8 kb *SalI* fragment of *Hsp70a* cloned in pUC18 (pCB353) was kindly provided by Prof. Christoph Beck, University of Freiburg, Germany (von Gromoff and Beck, 1993). Plasmid CS2.1 that

contains an *EcoRI* fragment of the gene of the small subunit of ribulose biphosphate-carboxylase (*RbcS2*) was kindly provided by Michel Goldschmidt-Clermont, University of Geneva, Switzerland. This probe hybridizes to both *RbcS1* and *RbcS2* genes of *C. reinhardtii* (Goldschmidt-Clermont and Rahire, 1986). Probes for the *Fesod* (Chen *et al.*, 1996) and *CabII-1* (Imbault *et al.*, 1988) genes were obtained by PCR and cloned into pGEM-T Easy (Promega). The 323 bp *Fesod* fragment was obtained in a PCR with the primers 5'-accgcgcctacgtggataac-3' and 5'-cagcttgccggtctgttcg-3' at an annealing temperature of 56 $^{\circ}$ C, and the 413 bp *CabII-1* fragment was obtained by PCR with the primers 5'-ggcaccaagtctcgcgaggctgtc-3' and 5'-gtggcgaaggcgaaggcgtgttg-3' at an annealing temperature of 67 $^{\circ}$ C.

RNA preparation and hybridization

Total RNA was prepared by the acid guanidine isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) with TRIzol Reagent (Life Technologies) following the supplier's instructions.

Aliquots of 20 μ g total RNA were denatured with glyoxal/DMSO, fractionated on agarose gels, blotted, hybridized and washed following the protocol of Ausubel *et al.* (1994). The final wash in 0.1 $\times$ SSC and 0.1% SDS was carried out at 65 $^{\circ}$ C. The amount of total RNA was checked by ethidium bromide staining

and verified by hybridizing the same blots with *RbcS* or *CabII-1* cDNA probes.

Reporter gene constructs

A 1300 bp *EcoRV*-*Clal* promoter fragment that included 51 bp of the transcribed sequence of *Gpxh* was cut from a chromosomal clone of *Gpxh* and was cloned into the *Clal*/*StuI* sites of pUC28. This construct was cut with *EcoRV* and *SalI* and the resulting promoter fragment was cloned into *SalI*- and *EcoRV*-digested pJD54. The resulting construct was designated pASPro1 (Davies *et al.*, 1992; Quinn and Merchant, 1995) (see Figure 6). The shorter constructs pASPro2 and pASPro3 were obtained by PCR (annealing temperature 56 °C) on pASPro1 with the upstream *KpnI*-site-containing primers 5'-aaggtaccacagcaggtgatgggtgagtct-3' and 5'-taggtaccagccgatggagaggagcactt-3' located in the *Gpxh* promoter region and downstream primer 5'-atcttgatggttcgtcctgagc-3' located on the *Ars* gene. The PCR fragments were cloned into pGEMTeasy (Promega). Upon digestion with *KpnI* and *EcoRV*, fragments were isolated and cloned by exchanging the *KpnI*-*EcoRV* fragment of pASPro1. For the construction of pASPro4,5,6, a similar PCR approach (annealing temperature 64 °C) was chosen using the same downstream primer, but without the *KpnI* site in the upstream primers 5'-cttttcgctgttgctgtgtgg-3', 5'-ggatgatgacaccgcccgcga-3', 5'-acaccgcccgcagttgagc-3'. The PCR products were cloned into pGEMTeasy, re-isolated upon digestion with *EcoRV* and *SalI* and cloned into *EcoRV*- and *SalI*-digested pASPro1, giving the plasmids pASPro4, pASPro5 and pASPro6. For the construction of plasmid pASPro2ΔCRE, with a deleted CRE-like sequence in the *Gpxh*-*Ars* reporter, a PCR-based method on pASPro1 was used. In a first PCR, 422 bp upstream of the CRE-like sequence were amplified with a *KpnI*-containing upstream primer (5'-aaggtaccacagcaggtgatgggtgagtct-3') and a primer that binds immediately upstream from the CRE-like sequence and contained a *XhoI* restriction site (5'-ggctcgaggacgttgccgcacacctac-3'). In a second PCR, a 265 bp fragment was obtained using an upstream primer with a *XhoI* restriction site (5'-ggctcgagggttagagaagccaagttg-3'), annealing immediately downstream from the CRE-like sequence and a downstream primer that annealed in the *Ars* sequence (5'-atcttgatggttcgtcctgagc-3'). Both the 422 and 264 bp fragments were cloned into pGEMTeasy, yielding plasmid pG422 and pG264 respectively. A

264 bp *NcoI*-*XhoI* downstream fragment was sub-cloned into *NcoI*-*XhoI*-digested pG422. Finally, a *KpnI*-*EcoRV* CRE-deleted promoter fragment was isolated from this plasmid and used to exchange the *KpnI*-*EcoRV* promoter fragment of pASPro1. The clones pJD54 and pJD55 were generously provided by J. Davies.

Strain cw_{15arg7} was co-transformed with reporter gene constructs and pT7-Arg7 (Auchincloss *et al.*, 1999) or pArg7.8 (Debuchy *et al.*, 1989) following the protocol of Kindle (1990). Transformants were selected on TAP agar plates without arginine, and the colonies were screened for *ars* expression by spraying the plates with X-SO₄ (5-bromo-4-chloro-3-indolyl sulfate potassium salt) dissolved in water to give a final concentration of 0.05 mM on the plates (Biosynth).

ARS assay

Quantitative assays of ARS activity were performed essentially as described by de Hostos and colleagues (De Hostos *et al.*, 1988, 1989; Davies *et al.*, 1992). Cultures of individual clones were grown in TAP to a density of ca. 3×10^6 cells/ml. To remove the ARS excreted into the medium during growth, the cells were harvested by centrifugation at $425 \times g$ at 4 °C for 10 min, and resuspended in TAP at 20×10^6 cells/ml. The culture was distributed into different fractions for exposure experiments. For each ARS activity measurement, 300 µl of culture was centrifuged at maximal speed for 2 min, 100 µl of the supernatant was mixed with 85 µl of a buffered substrate solution containing 0.2 M glycine (from a 1 M glycine-NaOH pH 9.0 stock), 20 mM imidazole, and 9 mM *p*-nitrophenylsulfate (Fluka) (GIN solution). The mixture was incubated for 10 min at 30 °C, the reaction was stopped with 750 µl 0.5 M NaOH, and the absorbance at 410 nm was measured.

Results

Gpxh mRNA levels strongly increase upon exposure to ¹O₂ photosensitizers and organic hydroperoxides but exhibit different induction kinetics

Recently we reported the isolation and characterization of a glutathione peroxidase homologous gene (*Gpxh*) in *C. reinhardtii* and showed that *Gpxh* mRNA levels were up-regulated upon oxidative stress rather than upon general stress conditions in *C. reinhardtii* (Leisinger *et al.*, 1999). To obtain more insight into

Figure 3. Expression of *Gpxh* in the presence of photosensitizers in the light and in the dark. Northern blot analysis of RNA extracted before addition and 30 min and 3 h after addition of the photosensitizers neutral red (NR), methylene blue (MB) and rose Bengal (RB) at the concentrations indicated in the light or in the dark. From cultures that died during the run of the experiment, only the RNA extracted 30 min after exposure was blotted.

the function of *Gpxh* and examine its regulated expression, we investigated the kinetics of *Gpxh* mRNA concentration patterns in *C. reinhardtii* cells in more detail after exposure to a variety of treatments that cause oxidative stress.

Northern blot analysis of total RNA extracted from *C. reinhardtii* cells that had been exposed to different concentrations of hydrogen peroxide, as well as the superoxide radical-generating chemical paraquat, showed a weak induction only at sublethal (2 mM H_2O_2) or lethal (5 μM paraquat) concentrations (Figure 1). Induction was slow and mRNA levels were higher after 3 h than after 30 min exposure. At 10% of the sublethal concentrations, virtually no increased *Gpxh* mRNA levels were observed. Similar results were obtained in experiments with the superoxide radical-generating chemicals juglone and menadione (data not shown). In cultures exposed to the organic hydroperoxide *tert*-butyl hydroperoxide (t-BOOH), significantly raised *Gpxh* levels were observed be-

tween 0.06 mM and 2 mM (lethal concentration), whereas only a small increase was observed at lower levels (Figure 1). Effects on the *Gpxh* mRNA levels upon exposure to cumene hydroperoxide (CU-OOH), an alternative organic hydroperoxide, were similar to the t-BOOH exposure (data not shown). Interestingly, exposure to various concentrations of the singlet oxygen ($^1\text{O}_2$)-generating photosensitizer neutral red (NR) caused a fast and very strong increase in *Gpxh* mRNA levels, even at 1% of the lethal concentration (Figure 1). The *Gpxh* mRNA concentration levelled off in the 3 h sample but remained higher than that observed in the untreated culture. These results suggest that the strong increase in *Gpxh* mRNA levels upon exposure to NR is not caused by oxidative stress in general, but specifically by the presence of $^1\text{O}_2$. The differences in induction kinetics upon exposure to organic hydroperoxides and NR became clearer in a more extensive time course (Figure 2). In cultures where NR was added, the increase in *Gpxh* mRNA level started

Figure 4. Expression of *Gpxh* upon a dark-light shift in the strain *cw15arg7*. The cells were grown in the light and dark-adapted for 2 h. One third of the culture was kept in the dark, one third was shifted to the light ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation, PAR), and one third was shifted to the light and incubated in presence of $5 \mu\text{M}$ of the $^1\text{O}_2$ photosensitizer neutral red (NR). Total RNA was extracted from cultures before dark-light shift and at 30 min and 3 h after the shift. A $20 \mu\text{g}$ portion of total RNA from each sample was used for a northern blot and hybridized with *Gpxh*.

after 7 min and reached its maximum after 20–40 min. In cultures to which the organic hydroperoxide CU-OOH had been added, a significant increase in mRNA was not visible before 40 min exposure and levels remained raised at least until 80 min.

An $^1\text{O}_2$ dependence of *Gpxh* expression was further supported by exposing *C. reinhardtii* cells to other $^1\text{O}_2$ -generating photosensitizers, rose Bengal (RB) and methylene blue (MB). Again, a fast and strong increase in *Gpxh* mRNA levels was observed (Figure 3). The three $^1\text{O}_2$ -producing photosensitizers NR, RB and MB are very potent inducers since they resulted in strongly increased *Gpxh* mRNA levels when administered in lethal ($10\text{--}100 \mu\text{M}$) and sublethal concentrations and even at levels far below the lethal concentration.

Photosensitizers exposed to light in the presence of molecular oxygen give rise to the generation of singlet oxygen ($^1\text{O}_2$), whereas no $^1\text{O}_2$ is generated in the dark. In control experiments in which *Gpxh* mRNA levels were analysed from dark-incubated cultures to which the same concentration of the photosensitizers had been added, no increased *Gpxh* mRNA levels were observed (Figure 3). This strongly suggests that the induction of *Gpxh* transcription is a result of the photosensitized $^1\text{O}_2$ production by these compounds, rather than the result of light-independent reactions of the compounds.

Gpxh mRNA levels increase upon enhanced physiological $^1\text{O}_2$ production

To support the significance of the $^1\text{O}_2$ -induced rise in *Gpxh* mRNA levels, *Gpxh* mRNA profiles were examined under conditions that lead to increased $^1\text{O}_2$ formation inside cells by natural photosensitizers or photosynthesis. In a shift of a dark-adapted culture

to the light, the $^1\text{O}_2$ generation inside cells increases due to the photosensitizer action of biomolecules and due to photosynthetically produced $^1\text{O}_2$ in *C. reinhardtii* (Roudyk *et al.*, 1996). In the light, the mRNA concentrations of *Gpxh* strongly increased upon exposure to light after 30 min and 3 h, whereas no significant change occurred in the mRNA levels of the dark-adapted cultures (Figure 4). Light caused a fast induction of the *Gpxh* gene, resembling the induction kinetics after exposure to photosensitizers.

In another approach, the effect of increased protoporphyrin IX levels was studied. Protoporphyrin IX is a universal intermediate in the synthesis of heme, bilins and chlorophylls generated in the chloroplast (Timko, 1998) and is a potent singlet-oxygen-generating photosensitizer (Devine *et al.*, 1993; Ryter and Tyrrell, 1998). Several mutants of *C. reinhardtii* have been described that are blocked in the formation of Mg-protoporphyrin IX from protoporphyrin IX (CC-52, CC-339, CC-2398) and therefore accumulate large amounts of protoporphyrin IX (Stolbova, 1971; Wang *et al.*, 1974; Timko, 1998). Mutants were pre-grown under very low light ($<1 \text{ mmol}$ photosynthetically active radiation (PAR) $\text{m}^{-2} \text{s}^{-1}$), divided into a portion that was kept in the dark, a second portion that was shifted to the light and a third portion that was shifted to the light with the concomitant addition of $10 \mu\text{M}$ NR. In two mutants, CC-2398 (*chl-1-19*, *pf-14*; Stolbova, 1971) and CC-339 (*brs-1*, *r-1*; Wang *et al.*, 1974), the dark-light shift resulted in a fast and strong increase in *Gpxh* transcript levels much higher than those in the wild-type cells (data not shown). Additional NR did not increase the *Gpxh* mRNA amount in the mutant cells much further. We conclude that in these mutants the accumulated protoporphyrin IX probably caused the generation of $^1\text{O}_2$ which triggered enhanced *Gpxh* expression. The mutant CC-52 which, like strain CC-339, accumulates protoporphyrin IX due to a *brs-1* mutation, showed a different behaviour. The dark-light shift caused an increase in *Gpxh* transcript levels that was even less than in the wild-type strain. Since the addition of NR resulted in a strongly increased *Gpxh* mRNA level, as in the wild-type cells, a failure in the $^1\text{O}_2$ signalling pathway could be excluded (data not shown). Mutation *chl-1-19* of CC-2398 is supposed to be allelic to *brs-1*. Mutation *pf-14* influences motility and should therefore not interfere with the $^1\text{O}_2$ response (Timko, 1998). Hence, we expected equal behaviour of the strains CC-52 and CC-2398, which is not the case for unknown reasons. The CC-339 cells contain the additional *r-1* mutation

Figure 5. Expression of *Gpxh* upon a dark-light shift in nitrofen-treated *cw15arg7* cells. The cells were grown overnight in the complete dark in the presence of nitrofen. Fractions of these cultures were exposed to light, while other fractions were kept in the dark. Total RNA was isolated and used for northern blots. *Gpxh* was used as a probe.

that leads to increased accumulation of protoporphyrin IX for reasons not yet known. Thus, a different behaviour of this mutant as compared to strain CC-52 was expected, but this was not the case (Wang *et al.*, 1974).

In order to avoid differences due to secondary mutations or genetic backgrounds of the strains used, strain *cw15arg7* was grown in complete darkness in the presence of nitrofen, which causes the accumulation of protoporphyrin IX (Devine *et al.*, 1993). Subsequently, fractions of the cultures were exposed to light while other fractions remained in the dark. Addition of 10^{-5} and 10^{-4} M nitrofen caused increased *Gpxh* mRNA levels. Lower nitrofen concentrations did not lead to a significantly increased *Gpxh* mRNA level in comparison with the untreated cells (Figure 5). The induction was caused by a photochemical reaction as no elevated *Gpxh* levels were observed in the dark-incubated controls. Under all conditions applied, the cells remained green, indicating that chlorophyll synthesis was not completely inhibited by nitrofen up to 10^{-4} M. In a second series of experiments, with up to 10^{-3} M nitrofen, the cells turned brown and the same induction pattern was observed (data not shown). We conclude that the protoporphyrin IX that accumulates due to nitrofen treatment triggers increased *Gpxh* expression, probably through the formation of $^1\text{O}_2$.

Induction of *Gpxh* shows higher specificity for $^1\text{O}_2$ than induction of other stress-responsive genes

Northern blots in Figure 1 were hybridized with two other stress-responsive genes: (1) the cytosolic heat shock protein HSP70A encoding gene (*Hsp70a*) and (2) the nuclear-encoded iron-containing superoxide dismutase gene (*Fesod*) that codes for the chloroplast-localized FeSOD (von Gromoff *et al.*, 1989; Chen *et al.*, 1996; Figure 1). This was done in order to check whether the strong and fast increase in *Gpxh* mRNA levels by $^1\text{O}_2$ is specific for this gene and thus related

to the function of GPXH, or whether its induction is rather a general stress response that is similar for other stress-responsive genes. The *Hsp70a* induction pattern by NR and t-BOOH was similar to *Gpxh* induction in that (1) induction occurred at the same concentrations, (2) the induction by NR occurred fast (higher mRNA concentrations after 30 min than after 3 h) and (3) the induction by t-BOOH was rather slow (higher mRNA concentrations after 3 h than after 30 min). However, induction of the *Hsp70a* gene by H_2O_2 was clearly different from the *Gpxh* induction. The *Hsp70a* mRNA concentration reached higher relative levels at lower H_2O_2 concentrations and the mRNA levels were higher at 30 min than at 3 h in contrast to the slower *Gpxh* mRNA accumulation. These results indicate differences in the regulation of mRNA concentrations of *Hsp70a* and *Gpxh*. The induction pattern of *Fesod* mRNA was completely different to that of both *Hsp70a* and *Gpxh*. H_2O_2 and paraquat induced at least as strongly as t-BOOH and NR, lower concentrations of NR (1 μM) only caused very weak inductions, and the *Fesod* mRNA concentrations were about the same at 30 min and 3 h after addition of the compound. The *Fesod* mRNA responses were less specific, weaker and slower upon NR addition than the responses of both *Gpxh* and *Hsp70a* mRNA.

Thus, *Gpxh*, *Hsp70a* and *Fesod* all show induction upon different types of oxidative stress, but the mRNA induction patterns are clearly distinct from gene to gene and from one type of stress to the other. Among the genes investigated, *Gpxh* is most specifically induced by $^1\text{O}_2$ photosensitizers. The photosensitizers cause a very strong induction even at low concentrations, and the relative induction by other compounds investigated is much weaker for the *Gpxh* mRNA than for the other genes examined in this study.

Figure 6. Reporter gene constructs and activities. **A.** Plasmid constructs containing *Gpxh* promoter fragments fused to *Ars* reporter gene, used for transformation of *cw15arg7* cells. Numbers indicate the relative position to the transcription start in the *Gpxh* promoter. Boxes indicate special promoter fragments: CRE, cAMP-responsive element-like sequence; G, putative GC box; C, putative CAAT boxes. Unique restriction sites are underlined. **B.** A number (*n*) of individual clones of the different transformants were tested in induction studies. Cells were washed, resuspended in TAP and grown for an additional 8 h under illumination before the determination of ARS activity. Where indicated, 10 μ M NR or 0.2 mM t-BOOH was added to the cultures during this time. To examine the effect of protoporphyrin IX accumulation, cultures were grown in the dark in the presence of 10⁻⁴ M, 10⁻⁵ M or 10⁻⁶ M nitrofen and subsequently shifted to 80 μ mol m⁻² s⁻¹ photosynthetically active radiation (PAR). To assess the effect of a dark-light shift, cultures have been pre-incubated in the light and subsequently transferred to 80 μ mol m⁻² s⁻¹ PAR. Induction fold (arylsulfatase activity in the induced culture divided by the activity in the unexposed culture) was calculated and averaged for each transformant. Averaged values are presented for the different constructs.

Gpxh mRNA levels are regulated by transcriptional activation ($^1\text{O}_2$)

A reporter gene approach was chosen to (1) investigate the regulation of the *Gpxh* gene in more detail, (2) determine promoter elements that are involved in the singlet-oxygen-induced *Gpxh* activation, and (3) gather insight into the regulatory pathway leading to the induction. A series of constructs were made in which *Gpxh* promoter fragments of various length were cloned in front of the promoterless arylsulfatase reporter gene (*Ars*) and were transformed into *cw15arg7* (Figure 6, panel A) (de Hostos *et al.*, 1988, 1989; Davies *et al.*, 1992; Ohresser *et al.*, 1997). For control experiments the same strain was transformed with plasmid pJD55 that contains the *Ars* reporter gene under the control of the constitutive β -tubulin promoter (Davies *et al.*, 1992). For each construct, different individual transformants were exposed to light in the absence (control) or presence of the singlet-oxygen photosensitizer NR and tested for singlet-oxygen-responsive accumulation of arylsulfatase activity. Constructs pASPro1, pASPro2, pASPro3 and pASPro4 containing the *Gpxh* promoter region from -1.3 kb, -562 bp, -355 bp and -179 bp respectively to $+51$ bp relative to the transcription start revealed a strong NR-responsive expression of the reporter gene (Figure 6, panel B). Differences in expression levels were observed between individual clones with the same construct. Such differences arise as a result of the number and site of the chromosomal integrations, as well as the degree of gene silencing (Mayfield, 1991; Blankenship and Kindle, 1992; Cerutti *et al.*, 1997; Kindle, 1998). With constructs pASPro5 and pASPro6, having shorter promoter pieces (-104 bp and -96 bp relative to $+51$ bp), only a slight increase in *ARS* expression by NR was observed. Increased marker gene expression upon NR exposure was never observed with *C. reinhardtii* transformants with the control plasmid pJD55. Generally a decreased expression was observed, probably reflecting the toxic effects of NR.

These data indicate that a singlet-oxygen-responsive region is located between nucleotides -104 and -179 , found upstream of the putative promoter GC box (-93 to -88) and CAAT boxes (-77 to -74 and -47 to -44). Within this region, a sequence (5'-TGACGCCA-3') has been identified that is homologous to cAMP-responsive elements (CRE) in mammals (5'-TGACGTCA-3') and, although less pronounced, to the activator protein 1 (AP-1) binding

site (5'-TGACTCA-3'). In the *Gpxh* promoter, this sequence is part of a 16 bp long, almost perfect palindrome (5'-GCGCCAACGTTGACGC-3'). This palindrome resembles, with only two symmetric mismatches, the palindromic canonical motif 5'-CNNGAANNTTCNNG-3' of heat shock elements, *HSE* (Bienz and Pelham, 1986). The *HSEs* identified in the *hsp70a* promoter of *C. reinhardtii* also contain two mismatches each, but are nevertheless functional (Müller *et al.*, 1992; Kropat *et al.*, 1995). However, the *Gpxh* mRNA was only slightly up-regulated by a heat shock incubation at 40°C (unpublished data). In construct pASPro2 Δ CRE, in which the CRE-like sequence was deleted, induction upon exposure to NR was decreased. This substantiates the importance of this sequence in $^1\text{O}_2$ -induced *Gpxh* expression. The involvement of other regulatory sequences can, however, not be excluded at this stage.

Since all constructs contain the first 55 bp of the 5'-untranslated region (5'-UTR) of the *Gpxh* mRNA, we cannot exclude that the 5'-UTR is involved in the accumulation of arylsulfatase activity.

To further support the role of $^1\text{O}_2$ in the transcriptional induction of *Gpxh*, transformants containing construct pASPro1 were (1) incubated in the dark and subsequently exposed to light and (2) exposed overnight to nitrofen in the dark and then transferred to the light. Reporter gene expression was induced under both conditions (Figure 6, panel B). This confirms the results of the northern blots (Figures 4 and 5) and supports the role of $^1\text{O}_2$ in the transcriptional regulation of *Gpxh* expression.

Although exposure to t-BOOH caused a significant though slow increase in *Gpxh* mRNA levels in *C. reinhardtii*, no increased reporter gene expression was observed upon exposure of the transformants to t-BOOH (Figure 6, panel B). Northern blot experiments substantiated this finding in that almost no increase in reporter gene mRNA could be detected, whilst a strong increase of *Gpxh* mRNA concentration upon exposure to t-BOOH was found in the same samples (data not shown). Either all constructs lacked a t-BOOH-responsive element, or the increase of *Gpxh* mRNA upon t-BOOH exposure is not caused by transcriptional up-regulation but by increased mRNA stability.

Discussion

Our data suggest that $^1\text{O}_2$ is a specific trigger of *Gpxh* induction. The $^1\text{O}_2$ photosensitizers strongly

and quickly induced *Gpxh* mRNA with induction patterns that were independent of the photosensitizer used, despite the distinct chemical properties and bulk distributions of the photosensitizers within *C. reinhardtii* cells (Ettl, 1976; Clark, 1981; Kochevar *et al.*, 1994). Levels of the *Gpxh* mRNA significantly increased upon exposure to organic hydroperoxides, although the increase was much slower and sustained for a longer period of time. Reporter gene experiments indicated that the observed differences in kinetics are the result of different induction mechanisms. The fast increase triggered by $^1\text{O}_2$ photosensitizers is the result of transcriptional up-regulation, whereas the mechanism of the slow accumulation upon exposure to t-BOOH is not known and might be due to increased mRNA stability. Alternatively, the t-BOOH caused increases in *Gpxh* mRNA might also have been mediated by $^1\text{O}_2$ generated in reactions of organic hydroperoxides with iron centres, or through lipid peroxidation (Cadenas and Sies, 1998; Ryter and Tyrrell, 1998). This would cause a lag phase as well.

The *Gpxh* mRNA levels increased slightly upon exposure to various forms of sublethal oxidative stress, other than organic hydroperoxides and $^1\text{O}_2$. It is not clear whether this weak increase of *Gpxh* mRNA has to be assigned to a more general induction of defence genes under sublethal conditions, or to $^1\text{O}_2$ generated in oxidative chain reactions that were initiated by the applied chemicals.

The involvement of $^1\text{O}_2$ was further substantiated by the use of mutants that accumulate the photosensitizing protoporphyrin IX. In a complementary approach, protoporphyrin IX levels were raised in nitrofen-treated wild-type cells. Nitrofen-treated wild-type cells and the two mutants, CC-339 and CC-2389, showed higher *Gpxh* mRNA levels after a dark-light shift than did wild-type cells. This is presumably caused by higher photosensitized $^1\text{O}_2$ formation. In contrast, the *brs-1* mutation in strain CC-52 caused reduced *Gpxh* mRNA levels upon dark-light shift as compared to wild-type cells.

Differences between inhibitor experiments and a *brs-1* mutant have been observed earlier and may be caused by a different genetic background (Jasper *et al.*, 1991). Secondary mutations that influence the expression of *Gpxh* under the conditions tested cannot be excluded. In all the mutants used, NR led to increased transcription levels as in the wild-type strains, indicating that the ' $^1\text{O}_2$ sensor' was intact in all of the strains.

Two lines of reasoning may be used against an $^1\text{O}_2$ -dependent regulation of the *Gpxh* mRNA levels: (1) the strong induction of *Gpxh* mRNA concentration by NR, MB, RB, and protoporphyrin IX could be triggered by a type of photosensitized reaction other than $^1\text{O}_2$ generation, or (2) the inducibility of *Gpxh* mRNA could be dependent on the localization of ROS inside the cell rather than on the type of ROS.

Although the specificity of the induction of *Gpxh* by photosensitizers is apparent, this does not necessarily indicate that $^1\text{O}_2$ is the trigger for induction. Photosensitizers in their excited triplet state not only can pass excitation energy onto molecular oxygen to form $^1\text{O}_2$, but they also have the common property of being easily oxidizable or reducible, thereby giving rise to superoxide radicals or a variety of organic radicals (Foote, 1991; Ryter and Tyrrell, 1998). An unspecific, irreversible redox reaction of the photosensitizers can be excluded because of the low concentrations required for induction. The irreversible, specific oxidation, or reduction of a critical molecule within the cell, can also be excluded because of the different chemical properties of the photosensitizers (Kochevar *et al.*, 1994). The photosensitizers may, however, undergo a light-driven redox cycling via their excited state and produce either reduced or oxidized radicals in reactions with biomolecules (Lambert and Kochevar, 1997; Stratton and Liebler, 1997). Such a redox cycling would result in changes of the pools of reduced and oxidized compounds (antioxidants and pro-oxidants) inside cells like ascorbate, glutathione or H_2O_2 , a situation known to trigger cellular responses (Foyer *et al.*, 1997). However, redox cycling is generally rejected for the action of RB, MB and other photosensitizers under aerobic conditions due to kinetic considerations and the analysis of reaction products (Lambert and Kochevar, 1997; Stratton and Liebler, 1997; Aveline *et al.*, 1998). It is also generally accepted that $^1\text{O}_2$ is a key intermediate in the actions of photodynamic dyes (Briviba *et al.*, 1997; Ryter and Tyrrell, 1998). In addition, similar changes in the pro-oxidant/antioxidant pools would result from reactions with superoxide radical, hydrogen peroxide and the resulting $\text{OH}\cdot$ or other ROS. However, incubation with such compounds did not cause any comparable induction of *Gpxh* transcription (Figure 1). In addition, the weak induction of *Gpxh* by H_2O_2 or paraquat might be explained by the fact that the ROS in these cases did not reach the decisive cellular compartment. H_2O_2 is an uncharged compound that can easily diffuse through membranes and sustains itself long enough

to reach any site inside the cell (Foyer *et al.*, 1997). Hence, site restriction can be excluded for H_2O_2 . Superoxide radicals usually occur in their anionic form $\text{O}_2^{\cdot-}$ and are unable to penetrate membranes (Halliwell and Gutteridge, 1989). The major electron donors for paraquat-mediated $\text{O}_2^{\cdot-}$ generation are localized to the chloroplast (Halliwell, 1984). Furthermore, superoxide, in general, is removed very efficiently in cells and its concentration stays below one molecule per cell (Devine *et al.*, 1993). Therefore, it is expected that locally generated superoxide will have local effects. However, a number of studies in *Escherichia coli* and other organisms have shown that the effect of superoxide generated by paraquat is not as local as expected (Halliwell and Gutteridge, 1989; Bray *et al.*, 1993). Therefore, we exclude superoxide as a possible inducer of *Gpxh* mRNA and conclude that $^1\text{O}_2$ triggers the strong increase of mRNA upon exposure to photosensitizers. It is unclear whether the induction of *Gpxh* was dependent on the localization of the generation of $^1\text{O}_2$ inside the cells. This potential dependence is implicated by the short average diffusion path length of $^1\text{O}_2$ in biological media (introduction) (Sies and Menck, 1992; Ehrenberg *et al.*, 1998; Ryter and Tyrrell, 1998). Furthermore, the carotenoids located in the thylakoids and chloroplast envelope quench $^1\text{O}_2$ efficiently and decrease the exchange of $^1\text{O}_2$ between chloroplast and cytosol (Timko, 1998). However, Roudyk *et al.* (1996) have shown that $^1\text{O}_2$ generated during photosynthesis in *C. reinhardtii* cells can be measured in the suspending medium, indicating that at least a fraction of the $^1\text{O}_2$ generated in the chloroplast can reach any location within the cell.

Singlet-oxygen-responsive element

The reporter gene experiments localized a singlet-oxygen-responsive promoter region from -179 to -104. Within this region, a sequence that is homologous to the mammalian cAMP response elements (CRE) and activator protein 1 (AP-1) binding site was identified as part of a 16 bp long palindrome. The importance of this CRE/AP-1 sequence for the singlet-oxygen-regulated *Gpxh* expression was shown by deleting this sequence. The identification of a putative CRE/AP-1 element in the *Gpxh* promoter is of particular interest, as the promoter of the rat heme oxygenase gene (*HO-1* gene) also contains a CRE/AP-1 element (5'-TGACTTCAGT-3'), the target sequence of a cGMP-mediated response (Immenschuh *et al.*, 1998). The mammalian *HO-1* gene is one of the

best investigated $^1\text{O}_2$ -up-regulated genes (Ryter and Tyrrell, 1998). However, to our knowledge, it has not yet been investigated whether the $^1\text{O}_2$ response of the *HO-1* gene is mediated by the CRE/AP-1 element. The surprising analogy between the two promoters prompted us to wonder whether aspects of the $^1\text{O}_2$ response could be conserved from mammals to plants, and thus if cyclic nucleotides may be involved in the response of *Gpxh* to $^1\text{O}_2$. Cyclic GMP seems to be of general importance in light-mediated signal transduction and ion channel regulation in plants (Brown and Newton, 1992; Bowler *et al.*, 1994; Penson *et al.*, 1996). Further investigations are required to determine the secondary messengers involved in $^1\text{O}_2$ signal transduction, and to investigate possible conserved traits between animals and plants.

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