Function and regulation of the glutathione peroxidase homologous gene *GPXH/GPX5* in *Chlamydomonas reinhardtii*

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Abstract When exposed to strong sunlight, photosynthetic organisms encounter photooxidative stress by the increased production of reactive oxygen species causing harmful damages to proteins and membranes. Consequently, a fast and specific induction of defense mechanisms is required to protect the organism from cell death. In *Chlamydomonas reinhardtii*, the glutathione peroxidase homologous gene *GPXH/GPX5* was shown to be specifically upregulated by singlet oxygen formed during high light conditions presumably to prevent the accumulation of lipid hydroperoxides and membrane damage. We now showed that the GPXH protein is a thioredoxin-dependent peroxidase catalyzing the reduction of hydrogen peroxide and organic hydroperoxides. Furthermore, the *GPXH* gene seems to encode a dual-targeted protein, predicted to be localized both in the chloroplast and the cytoplasm, which is active with either plastidic TRXy or cytosolic TRXh1. Putative dual-targeting is achieved by alternative transcription and translation start sites expressed independently from either a TATA-box or an Initiator core promoter. Expression of both transcripts was upregulated by photooxidative stress even though with different strengths. The induction required the presence of the core promoter sequences and multiple upstream regulatory elements including a Sp1-like element and an earlier identified CRE/AP-1 homologous sequence. This element was further characterized by mutation analysis but could not be confirmed to be a consensus CRE or AP1 element. Instead, it rather seems to be another member of the large group of TGAC-transcription factor binding sites found to be involved in the response of different genes to oxidative stress.

Introduction

In photosynthetic organisms, exposure to high light intensities can overwhelm the capacity of the photosynthetic electron transport chain and result in the uncontrolled production of reactive oxygen species (ROS). As a consequence, efficient defense systems evolved in plants and algae to prevent ROS formation, like the dissipation of excess light energy as heat by non-photochemical quenching or the enzymatic and nonenzymatic detoxification of ROS (Apel and Hirt 2004; Li et al. 2009). However, after prolonged exposure to high light, ROS like superoxide radicals (O_2^-) and singlet oxygen ($^1O_2$) can still be formed and result in the modification of proteins and DNA or in the initiation of lipid peroxidation causing irreparable damage and cell death. Thus, to avoid such harmful effects, repair mechanisms are induced. This includes enzymes like proteases to remove damaged proteins or peroxidases

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involved in the degradation of modified lipids (Thomas et al. 1990).

Glutathione peroxidases (GPX) are enzymes involved in the detoxification of hydrogen peroxide (H_2O_2) and organic hydroperoxides by reducing them to water and the corresponding alcohol. Two main classes of GPX have been identified. The first class contains a selenocysteine (SeCys) in its active site and exhibits strong peroxidase activity using glutathione (GSH) as reductant (Ursini et al. 1995). Nonselenium GPXs (NS-GPX), on the other hand, do not contain SeCys and are the only GPXs found in higher plants so far (Herbette et al. 2007). Most NS-GPX, but also some SeCys-GPX, lack a stretch of specific amino acids required for oligomerization (Ursini et al. 1995). This classifies them in a group of monomeric GPX called phospholipid hydroperoxide-GPX (PHGPX) due to their ability to directly remove organic hydroperoxides from lipid membranes (Maiorino et al. 1991; Thomas et al. 1990). Despite their classification as GSH-peroxidases, all plant NS-GPX characterized so far show higher activity using thioredoxin (TRX) as electron donor (Herbette et al. 2002; Iqbal et al. 2006; Jung et al. 2002; Navrot et al. 2006) showing the ubiquitous requirement of TRX as reductant. Thus, since the catalytic mechanism of NS-GPX is similar to the one described for some peroxiredoxins requiring TRX, it was suggested to classify these enzymes as a novel class of peroxiredoxins (Herbette et al. 2007; Rouhier and Jacquot 2005; Tanaka et al. 2005).

Unlike in higher plants, in the green alga *Chlamydomonas reinhardtii* both SeCys-containing and NS-GPXs have been identified (Dayer et al. 2008). The GPX-homologous gene *GPXH* (also called *GPX5*) is one of the three NS-GPX of *Chlamydomonas* and belongs to the PHGPX group of GPXs (Dayer et al. 2008). Its peroxidase activity has not been identified yet but a *GPXH* overexpression was shown to protect from oxidative stress-induced cell death (Ledford et al. 2007). *GPXH* was mainly studied for its specific induction by the increased formation of $^1O_2$ either produced by exogenous photosensitizers or during exposure to high light illumination (Fischer et al. 2004, 2006; Leisinger et al. 2001). It was postulated that the stimulation of *GPXH* expression by $^1O_2$ is a preventive response to combat lipid peroxidation after prolonged high light stress (Fischer et al. 2007a). Indeed, acclimation to low doses of $^1O_2$ formed by photosensitizers or high light treatment protected the cells from subsequent photooxidative stress and decreased lipid peroxidation (Fischer et al. 2007a; Ledford et al. 2007). Originally, the GPXH protein was predicted to localize in the cytoplasm. However, a recent comparison of expressed sequence tag (EST) sequences revealed that the *GPXH* gene might be expressed from two different transcription start sites resulting in a dual-targeted protein to the chloroplast and cytoplasm

(Dayer et al. 2008). Thus, the GPXH protein might be involved in the protection of two cellular compartments from oxidative damage. Still, neither the expression of *GPXH* from both transcription start sites nor the regulation of the two transcripts upon photooxidative stress has been examined experimentally yet.

Regulation of *GPXH* expression by $^1O_2$ was shown to require a 8 bp sequence element (TGACGCCA) homologous to the mammalian cAMP response element (CRE) and an activator protein-1 (AP-1) binding site in the promoter of the gene (Leisinger et al. 2001). However, this element has not been characterized further so far and it is not clear whether the 8 bp motif is sufficient to stimulate *GPXH* induction by $^1O_2$ or whether another element, a 16 bp palindrome overlapping the CRE/AP-1 element, is responsible for the response. In addition, other putative regulatory elements present in the *GPXH* promoter could be involved in the induction by $^1O_2$ too (Leisinger et al. 2001). The CRE-binding protein (CREB) was originally described to be activated by increased levels of cyclic AMP (cAMP) and over the years several different CRE-binding transcription factors have been identified (Daniel et al. 1998; De Cesare and Sassone-Corsi 2000; Mayr and Montminy 2001). They all belong to the activating transcription factor (ATF)/CREB family (Hai and Hartman 2001) some of which are activated by oxidative stress. The AP-1 transcription factor consists of the two proteins Jun and Fos, which either form a heterodimer of Jun and Fos or a homodimer of two Jun proteins to activate gene expression (Karin et al. 1997). AP-1 can be activated by ROS and the yeast AP-1 homolog YAP-1 was shown to be directly activated by H_2O_2 through interaction with the GPX3 protein (Delaunay et al. 2002). Thus, both CRE and AP-1 promoter elements can be involved in ROS signaling in mammals and might have a counterpart in *Chlamydomonas* which was found to have numerous similarities to animal systems (Merchant et al. 2007).

In this study, we performed a global analysis of *GPXH* in order to gain a broader view on the function and regulation of this gene in *C. reinhardtii*. We investigated the enzymatic activity of the expressed protein and addressed a putative function of the dual-targeted GPXH protein in the chloroplast and cytoplasm. Furthermore, the role of core promoter elements and various upstream regulatory elements on the expression of two independent transcripts of *GPXH* under photooxidative stress conditions were analyzed.

Materials and methods

Strains and culture conditions

Chlamydomonas reinhardtii strain $cw_{15}arg_7mt^-$ (CC-1618) transformed with corresponding plasmids was inoculated in

Tris–Acetate–Phosphate-medium (TAP) (Harris 1989) in Erlenmeyer flasks and agitated on a rotary shaker (150 rpm) under constant illumination (120 µmol m^{-2} s^{-1} PAR) at 25°C.

Escherichia coli DH5α (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 unmethylated pUC28. *E. coli* strains were grown on LB at 37°C.

Chemicals

The photosensitizers neutral red (NR) and rose bengal (RB) (Sigma) were dissolved in water and stored in 1 or 10 mM stock solutions at 4°C in the dark. The cyclic nucleotide analogs 8-bromoadenosine 3′:5′-cyclic monophosphate (8Br-cAMP) and 8-bromoguanosine 3′:5′-cyclic mono-phosphate (8Br-cGMP) (Sigma) (0.2 M stock solution in water) and the adenylate cyclase inhibitor MDL-12 (Calbiochem) (5 mM stock solution in water) were stored at −20°C. NADPH, glutathione, glutathione reductase (GR) from yeast, dithiothreitol, isopropyl-1-thio-β-D-galactopy-ranoside, cumene hydroperoxide (CUOOH), H_2O_2 and *tert*-butyl hydroperoxide (t-BOOH) were all purchased from Sigma.

Expression and purification of recombinant proteins in *E. coli*

The expression and purification of the *C. reinhardtii* TRXh1, m, x and y, the *Arabidopsis thaliana* TRXf and NADPH-dependent thioredoxin reductase b (NTRb) have been described elsewhere (Collin et al. 2003; Jacquot et al. 1994; Lemaire et al. 2003; Stein et al. 1995). For recom-binant *GPXH* expression, the cDNA of the short form of *GPXH* was cloned into the pET-8c-His expression vector (Leisinger et al. 1999) and transformed into *E. coli* BL21 (DE3) pLysS (Schenk et al. 1995). Transformants were grown in LB medium supplemented with 100 mg l^{-1} ampicillin at 37°C up to OD$_{600}$ = 0.6. *GPXH* expression was induced with 0.4 mM isopropyl thio-β-D-galactoside overnight at 30°C and cells were harvested by centrifuga-tion (5,000*g*, 15 min and 4°C). The pellet was resuspended in 30 mM Tris–HCl (pH 7.9) supplemented with 1 mM of the serine protease inhibitor phenylmethanesulphonyl-fluoride (PMSF) (Sigma). The cells were then disrupted by three passages through a French press (10,000 psi), and cell debris were sedimented by centrifugation (39,000*g*, 30 min, 4°C). The His-tagged protein products were affinity purified from cell extracts on Ni^{2+}-nitrilotriacetic acid resin (Sigma) by successive washing and eluting steps with increasing concentrations of imidazole (5–300 mM) in 30 mM Tris–HCl (pH 7.9) buffer. The presence of the purified recombinant protein was tested by SDS/PAGE for each fraction. The fractions containing the purified protein were dialyzed overnight against 5 l of 30 mM Tris–HCl (pH 7.9), 1 mM EDTA buffer and concentrated using a Centricon YM-3 (Millipore). Concentration of the purified protein was determined by measuring the absorbance at 280 nm using a molar extinction coefficient of 13,075 M^{-1} cm^{-1}, and aliquots were stored at −20°C.

In vitro peroxidase assays

GSH- and TRXh1-dependent peroxidase activity was measured in an NADPH-coupled spectrophotometric assay by monitoring the decrease in absorbance at 340 nm aris-ing from NADPH oxidation (Maiorino et al. 1990). The assay was carried out at 30°C in a 500 µl reaction mixture containing 30 mM Tris–HCl (pH 7.9), 1 mM EDTA, 180 µM NADPH, 0.145 µM to 3 µM of the recombinant GPXH. Then either 1.8 mM GSH and 0.5 U yeast GSH reductase (GR) for the GSH-system, 10 µM TRXh1 and 3 µM of the recombinant Arabidopsis NTRb for the TRX-system or nothing for the NADPH-system was added to the reaction mixture. The reagents were pre-equilibrated for 1 min and the reaction was started by adding the peroxide. Linear decreases in absorbance of NADPH and an absorption coefficient of 6.22 mM^{-1} cm^{-1} were used to calculate the enzymatic activity, corrected for GPX inde-pendent NADPH oxidation in peroxidase-free control reactions. The substrate preference of GPXH toward the peroxides H_2O_2, t-BOOH, and CUOOH was assessed in triplicates by measuring the TRX-dependent activity with 6 to 11 different peroxide concentrations (5 µM to 4 mM) and maintaining saturating concentration of the other components. Specific enzymatic activity for each peroxide concentration was determined and used to calculate the apparent kinetic parameters from non-linear regression with the Michaelis–Menten equation by IGOR software (WaveMetrics, Lake Oswego, USA).

In *C. reinhardtii*, chloroplastic TRXs are not reduced by a classical NADPH-dependent thioredoxin reductase but by a ferredoxin–thioredoxin light dependent reduction system (Lemaire et al. 2007). This system can be replaced by the chemical reductant dithiothreitol (DTT). In this case, the GPXH-dependent reduction of H_2O_2 with the recombinant chloroplastic TRXs is measured using the FOX colori-metric assay (Jaeger et al. 1994) detecting changes in peroxide concentrations by following the oxidation of ferrous to ferric ion with the ferric ion sensitive dye xylenol orange. This has to be done in the presence of 800 µM of DTT necessary to support the reduction of the TRXs (Navrot et al. 2006). Thus, the reaction mixture contained 2 µM of recombinant GPXH, 800 µM DTT, 10 µM recombinant purified TRX (Arabidopsis TRXf1 or TRXm,

x, y and TRXh1 from *Chlamydomonas*) and 800 μM H$_2$O$_2$ in 50 μl Tris–HCl buffer (30 mM, pH 7.9). After the reaction was started by adding the peroxide, the decrease of H$_2$O$_2$ content was detected over a period of 10 min by adding 5 μl of the reaction mixture to 1 ml of the FOX colorimetric reagent and measuring the absorbance at 560 nm after 15 min dark incubation.

Electrophoretic mobility shift assay

Crude extracts from *C. reinhardtii* cultures were prepared as described by Mittag ([1996](#)). Cells were grown to a density of 8×10^6 cells ml^{-1} and exposed to 5 μM NR for 1 h before harvesting. Radioactive DNA fragments from the *GPXH* promoter with the 8 bp CRE/AP-1 element were obtained by incubating 0.2 nmol of a 50 bases oligonucleotide in a 50 μl reaction mixture including 5 μl γ^{32}P ATP (370 mBq ml^{-1}) and 2 μl of Polynucleotide Kinase (Promega; 10 U μl^{-1}) for 1 h at 37°C. DNA was purified with a DNA purification kit (Qiagen) and annealed with 0.2 nmol of the corresponding unlabelled and complementary oligonucleotide by heating at 95°C for 1 min and subsequently incubating on ice.

Various amounts of crude cell extract were preincubated with 1 μg of poly d(I-C) in 16 μl for 20 min at 23°C to block unspecific DNA binding. Then 0.4 μl of the radiolabeled dsDNA fragment (Supplementary Table 1) was added and incubated for another 20 min at 23°C. After adding 3 μl of 5 × loading buffer (40% glycerol, 5 × TBE pH 8.3, 50 mM EDTA, 0.1% w/v bromophenolblue) the samples were separated on a 4% polyacrylamide gel containing 5% glycerol in 1× TBE (Ausubel [1994](#)). The gel was transferred to Whatman paper (3 mm), dried and exposed to an X-ray film for 10–48 h.

Construction and transformation of plasmids

Plasmids pASPro1, pASPro2 and pASPro2ΔCRE have been described before (Leisinger et al. [2001](#)). For construction of pASPro2 with the different mutations a 700 bp *Kpn*I–*Eco*RV promoter fragment of pASPro2 was subcloned in a *Kpn*I/*Eco*RV digested vector pT7blue (Novagen) resulting in plasmid pT7Pro5. Mutations were subsequently introduced with a site directed mutagenesis kit (Stratagene) either in the 8 bp CRE/AP-1 sequence element using primers 5′-CGCCAACGTTGACGCCT̲GTTAGAGAA-3′, 5′-CGCCA ACGTTGA̲G̲GCCAGTTAGAGAA-3′, 5′-CGCCAACGTT GACG̲T̲CAGTTAGAGAA-3′ or 5′-CGCCAACGTTGAC T̲C̲A̲GTTAGAGAA-3′ or in other putative promoter elements using primers 5′-CGCGGGATGATGACAC̲T̲T̲A̲T̲C GCAGTTGAGGCAATTCCCTGC-3′, 5′-ATGACACCGC CCGCAGTTGAGG̲T̲G̲C̲G̲TCCCTGCAGATGTTGA-3′,

5′-CAGATGTTGACGCGCTGGCT̲G̲C̲G̲TAGGAGTCTC TGTTATATAAAC-3′ or 5′-GGCTATTGAGGAGTCT CTGT̲G̲C̲G̲C̲TAAACCCTTTCACTCACATGC-3′, together with the corresponding reverse complement primer. For cloning of plasmid pASPro2ΔPal1 and pASPro2ΔPal2 the site directed mutagenesis kit (Stratagene) was applied with primer 5′-GCTGTGGTAGGTGTT̲C̲G̲A̲G̲GACGTTGACG CCAGTTA-3′ and plasmid pT7Pro5 as template or with primer 5′-GCTGTGGTAGGTGTT̲C̲G̲A̲G̲GACGTCCTCG AGGGTTA-3′ and a plasmid, containing the CRE-mutated *GPXH* promoter in pGEMTeasy (Promega) (Leisinger et al. [2001](#)) as template to introduce the mutations. All mutations were confirmed by sequencing. Finally, *Kpn*I–*Eco*RV fragments of each mutated promoter were isolated and used to exchange the 1.4 kb *Kpn*I–*Eco*RV promoter fragment of pASPro1.

The *TUBB2* promoter was excised from plasmid pJD55, kindly provided by J. Davis, as a 2.4 kb *Kpn*I–*Cla*I fragment and subcloned into *Kpn*I/*Cla*I digested pUC28. This construct served as template for PCR based introduction of the 8 bp CRE/AP-1 sequence element with and without flanking sequence into the *TUBB2* promoter. For plasmid pYSn2 the *TUBB2* promoter was amplified with the forward primer 5′-TGACGCCAGAGACGGCTTCCCGGCG CTG-3′ and the reverse primer 5′-GTGAGCGGATAAC AATTTCACA-3′, complementary to the pUC28 sequence using *Pfu* DNA polymerase (Promega) to generate blunt ended fragments. The same method was used to generate the *TUBB2* promoter fragments for pYSn3 and pYSn4 using primer 5′-GCGCCAACGTTGACGCCAGTTAGA GCTTCCCGGCGCTGCATG-3′ or primer 5′-GAGACG GCTTCCCGGCGCTG-3′ together with the same downstream primer. All three PCR products were digested with *Eco*RI and subcloned into *Sma*I and *Eco*RI digested pUC28 resulting in plasmid pYSn2p, pYSn3p and pYSn4p, containing the promoters for the final constructs. For plasmid pYSn5, pYSn6 and pYSn7, the *GPXH* promoter was amplified with primer 5′-TCAATATTATATAAACCCTT TCACTCACATG-3′ and 5′-GGTGACGAAGTACTGAG ACAGC-3′ from pASPro1, cut with *Ssp*I and *Sac*I and used to replace the *Eco*47III/*Sac*I fragments of plasmids pYSn2p, pYSn3p and pYSn4p, respectively. For pYSn1, finally, the *GPXH* promoter was amplified with primer 5′-ACGTTGACGCCAGTTAGAGAAG-3′ and 5′-GGTGAC GAAGTACTGA-GACAGC-3′ from pASPro1 and subcloned in the vector pPCR-Script™AmpSK(+) (Stratagene). All the modified promoter fragments were isolated by digesting the constructs with *Sal*I and *Cla*I and used to exchange the *Sal*I–*Cla*I promoter fragment of pASPro1.

Strain cw$_{15}$arg$_7$mt$^-$ was cotransformed with reporter gene constructs and 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 functional arylsulfatase expression by spraying the plates with 0.05 mM X-SO$_4$ (5-bromo-4-chloro-3-indolyl sulfate potassium salt, Biosynth AG) dissolved in water. Basal expression was detectable for all constructs and average expression varied between 0.31 and 1.41 relative to the pASPro2 wild-type construct.

Arylsulfatase assay

Quantitative assays of arylsulfatase activity were performed essentially as described earlier (Leisinger et al. 2001). Cells of individual clones were grown in TAP to a density of $\sim 5 \times 10^6$ cells ml^{-1}, harvested by centrifugation and resuspended in fresh TAP at the same density for all experiments. The culture was then distributed into different 5 ml fractions for exposure experiments in six well cell-culture plates. To induce the expression of reporter constructs 2 μM NR was used because this chemical does not bleach as fast as RB and is therefore better for long time exposure. Samples of 300 μl were taken after 2, 4 and 6 h of exposure and arylsulfatase activity was measured as described (Leisinger et al. 2001).

RNA isolation and quantitative real-time PCR

Cultures adjusted to 5×10^6 cells ml^{-1} were exposed to the condition indicated for 1 h before cells were harvested by centrifugation. Total RNA was isolated by the Trizol method as described earlier (Fischer et al. 2004). For quantitative real-time PCR (qPCR) experiments, 200 ng of individual total RNA was used in each 10 μl reverse transcription reaction with a reverse transcription kit (Applied Biosystems) according to the manufacturer's instruction.

Sequences of primers for qPCR were designed with the Primer Express^{TM} software (Applied Biosystems) and are shown in Supplementary Table 1. qPCR reactions were performed on the ABI Prism$^®$ 7000 Sequence Detection System (Applied Biosystems) as described earlier (Fischer et al. 2004). Threshold cycle (C_t) values were determined for all reactions in the logarithmic amplification phase, and the average C_t value was calculated for each sample out of three technical replicates. Amplification products were checked by gel electrophoreses and melting curves. Background C_t values of RNA without reverse transcription were determined and had to be at least six cycles more than C_t values of the corresponding cDNA. The efficiency for the amplification of each product was determined by serial dilutions of template cDNA and used to correct C_t values for variable amplification efficiencies. The C_t values of the 18S rRNA was used for normalization of variable RNA levels. Induction factors were calculated for each gene with the treated and the corresponding control sample in the same medium as an average with standard error out of three independent experiments.

Results

The *GPXH* gene is expressed as two independent transcripts predicted to encode a dual-targeted thioredoxin peroxidase
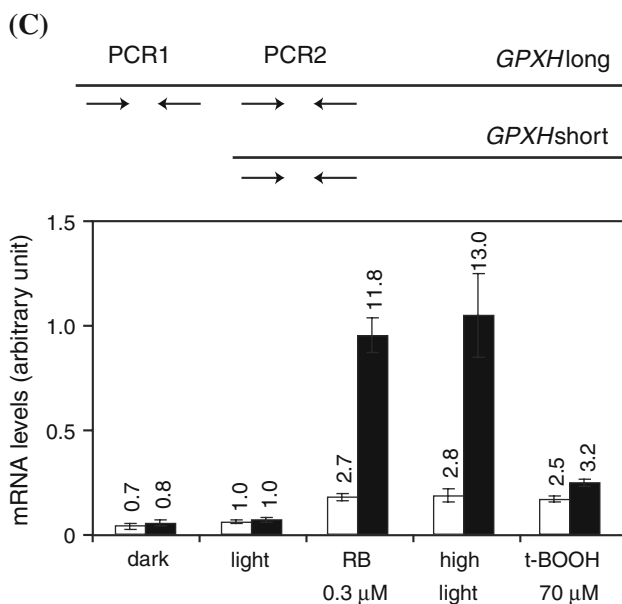
During the characterization of the *GPXH* gene the transcription start site was identified by primer extension (Leisinger et al. 1999). However, comparing this start site with the gene model based on EST sequences in the *Chlamydomonas* database of the Joint Genome Institute (JGI), another transcription start site was identified further upstream at position −45 compared to the originally identified start site. Indeed, a sequence element homologous to an alternative transcription start site called Initiator (Inr) (Smale 1997) was located close to this position (Fig. 1a). This longer transcript has an alternative translation start site which is 147 nucleotides upstream of the original ATG and gives rise to the same glutathione peroxidase homologous protein but with a 49 amino acid N-terminal extension (Fig. 1b). These additional amino acids were predicted by four out of five programs (ChloroP, PSORT, iPSORT, Predator) to code for a chloroplast transit peptide with a putative cleavage site at position 48 of the protein.

Transcription from independent start sites was tested by quantifying the expression of both the short (*GPXH*short) and the longer (*GPXH*long) transcript using a qPCR approach. Two different primer pairs were designed, of which one pair is specific to the 147 nucleotide extension of the *GPXH*long, whereas the other pair anneals to a region present in both transcripts (Fig. 1c). This allowed to quantify the amount of either *GPXH*long only or of both transcripts, enabling to calculate the amount of *GPXH*short by subtraction. By doing so, we could show that both transcripts are expressed to similar levels in dark and low light grown cultures (Fig. 1c). Upon exposure to photooxidative stress conditions caused by rose bengal (RB) and high light, *GPXH*short was strongly upregulated by 11.8- and 13-fold, respectively. The longer transcript *GPXH*long, on the other hand, was only induced by 2.7- to 2.8-fold under the same stress conditions. Treatment of cultures with the organic hydroperoxide *tert*-butylhydroperoxide (t-BOOH), shown to stimulate *GPXH* expression by mRNA stabilization (Fischer et al. 2007a), resulted in a similar increase in mRNA levels for both transcripts.

The predicted localization of GPXHlong in the chloroplast and GPXHshort in the cytoplasm raised the question whether the protein is functional in both compartments. Homology of the GPXH protein to peroxidases from other

(A)

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tggtaggtgtgcgccaacgttgacgccagt
tagagaagccaagtttgctaatcgcgggat
gatgacaccgcccgcagttgaggcaattcc
ctgcagatgttgacgcgctggctattgagg
agtctctgttatataaacccttttcactcac
ATGctgtctgcatacgcttgcggttcgcct
ttgcatctactgaaccagcgacgattgcaa
tcgattgcggtactcctgtcgccgctcgca
gcgtctgtcgcggtcgccaataaccaatcg
cctaacacctgtgctagcttcagcagcATG
```

(B)

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MLSAYACGSPLHLLNQRRLQSIAVLLSPLA
ASVAVANNQSPNTCASFSSMANPEFYG...
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(C)

◀ **Fig. 1 a** Sequence of the *GPXH* promoter presenting the position of the two independent transcription start sites (*arrows*), the translation start sites (*bold letters*) and the two core promoter elements TATA-box and Inr sequence (*underlined*). Homology searches resulted in the identification of five putative upstream regulatory elements (*grey boxes*) including the 16 bp palindrome (GCGCCAACGTTGACGC), the CRE/AP-1 binding site (TGACGCCA), the GC-box or Sp1 element (CCGCCC) and two CAAT boxes (CAAT and ATTG). Of these, three elements were shown in this study to be essential for induction by $^1O_2$ (*dark grey boxes*). **b** The N-terminal amino acid sequence of the longer GPXH protein is shown. *Bold letters* indicate the 49 amino acid extension predicted to function as a chloroplast transit peptide with the putative cleavage site at the position indicated by an *arrow*. **c** Expression of the short (*GPXH*short, *black bars*) and the long (*GPXH*long, *white bars*) transcript under different stress conditions was analyzed after 1 h by qPCR with primer pairs either only amplifying *GPXH*long cDNAs (PCR1) or all *GPXH* cDNAs (PCR2). *Numbers above bars* indicate fold changes of individual transcript compared to low light condition

Table 1 Steady-state parameters of the TRXh1-dependent peroxidase activity of GPXH were measured with different substrates

Substrate	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1} × 10^3)
H_2O_2	54 ± 5	7.4 ± 0.2	137 ± 49
t-BOOH	732 ± 112	11.3 ± 0.7	16 ± 6
CUOOH	63 ± 11	8.9 ± 0.4	141 ± 33

Michaelis–Menten constants (K_m), catalytic constants (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) were calculated as average ± SD from three independent non-linear regressions of the GPXH activity at increasing substrate concentrations (Supplemental Fig. 1)

organisms suggested that it might rather be a thioredoxin-dependent peroxidase than a glutathione peroxidase. To test this hypothesis the recombinant GPXH protein was over-expressed in *E. coli* (Supplementary Fig. 1A) and the peroxidase activity of the purified protein was tested using NADPH, GSH or cytosolic TRXh1 from *Chlamydomonas* as potential electron donors and H_2O_2, t-BOOH or cumene hydroperoxide (CUOOH) as substrates. Glutathione reductase or thioredoxin reductase were used when required (Gaber et al. 2001; Maiorino et al. 1990; Navrot et al. 2006). No activity was detected using GSH or NADPH with any of the peroxides tested (data not shown). With the cytosolic TRXh1, on the other hand, a strong reduction of all peroxides

was measured showing that the GPXH protein is a TRX-dependent peroxidase and potentially functional in the cytoplasm (Table 1). Analysis of Michaelis–Menten kinetics with increasing substrate concentrations (Supplementary Fig. 1B) revealed a more than tenfold higher K_m for t-BOOH than for H_2O_2 and CUOOH which resulted in a much lower catalytic efficiency (k_{cat}/K_m) for t-BOOH compared to the two other substrates (Table 1). To test a putative activity of GPXH in the chloroplast, four types of plastid TRXs (f, m, x and y) (Lemaire et al. 2003) were tested as electron donors, three from *Chlamydomonas* (TRXx, y and m) and one from Arabidopsis (TRXf). Reduction of H_2O_2 was quantified with a colorimetric FOX-assay (Jaeger et al. 1994). Indeed, with the chloroplastic TRXy the recombinant GPXH could reduce H_2O_2 at an even higher efficiency (98% reduction after 5 min) than with the cytosolic TRXh1 (53% reduction after 5 min) at an equimolar concentration (Fig. 2). In contrast, a much smaller decrease in H_2O_2 content (8–18% after 5 min) was observed with TRXx, m and f as electron donors. This thioredoxin specificity is consistent with the results previously reported for higher plant GPXs (Navrot et al. 2006).
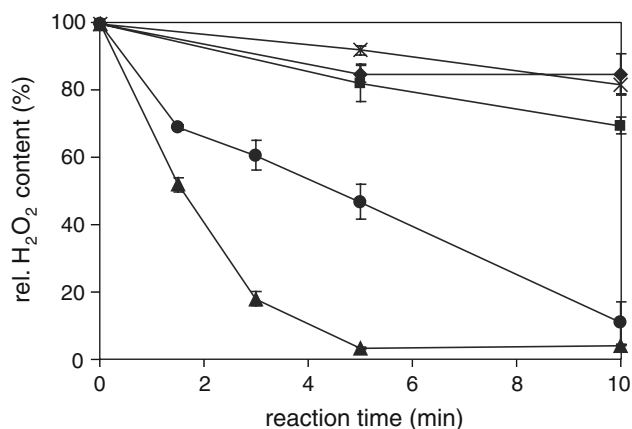
Fig. 2 Reduction of H_2O_2 by GPXH in the presence of cytosolic and chloroplastic TRXs was measured colorimetrically and expressed as a percentage of the initial H_2O_2 concentration, normalized to the activity without enzyme. *Chlamydomonas* cytosolic TRXh (●) and chloroplastic TRXm (■), TRXx (♦) and TRXy (▲) and Arabidopsis TRXf (×). Values of three independent measurements with SE are shown

Characterization of the CRE/AP-1 element involved in *GPXH* response to singlet oxygen

For transcriptional activation of *GPXH* expression by $^1O_2$ the presence of a CRE/AP-1 element (TGACGCCA) in the promoter was shown to be essential by removing the motif in a *GPXH*-arylsulfatase (*ARS*) reporter construct (pAS-Pro2ΔCRE) (Leisinger et al. 2001). However, this CRE/AP-1 element overlaps another putative *cis*-regulatory element consisting of a 16 bp palindrome (GCGCCAACGTTGA CGC). Therefore, we could not rule out that the reduced response of pASPro2ΔCRE was due to a mutation in the 3′-part of this palindrome. To test which of these elements is responsible for the response to $^1O_2$, two new reporter constructs were cloned where either only the 5′-part of the palindrome was mutated without affecting the CRE/AP-1 motif (pASPro2ΔPal1) or where the whole palindrome was exchanged (pASProΔPal2). Several independent transformants of each construct were tested to average for positional effects of the constructs after integration into the genome, and the reporter genes were analyzed for their induction by 2 μM neutral red (NR). Only a slightly lowered induction by NR compared to the wild-type construct (pASPro2) was found for pASPro2ΔPal1, whereas no induction could be measured with pASProΔPal2 (Fig. 3a). These results are consistent with those obtained with the pASPro2ΔCRE construct and indicate that indeed the CRE/AP-1 motif but not the palindrome is involved in the response to $^1O_2$.

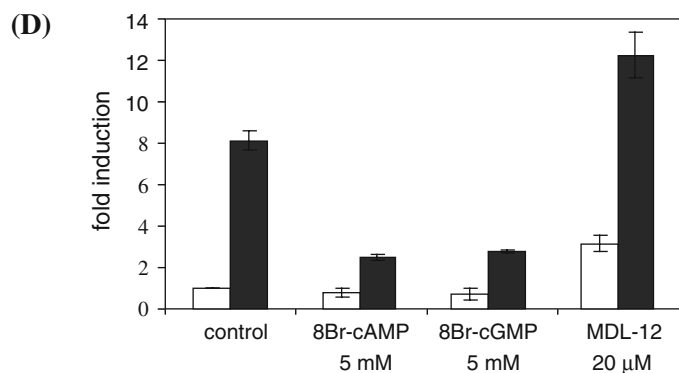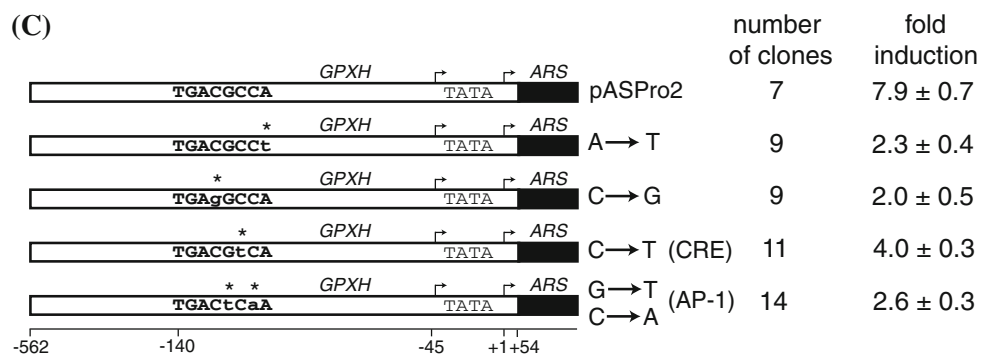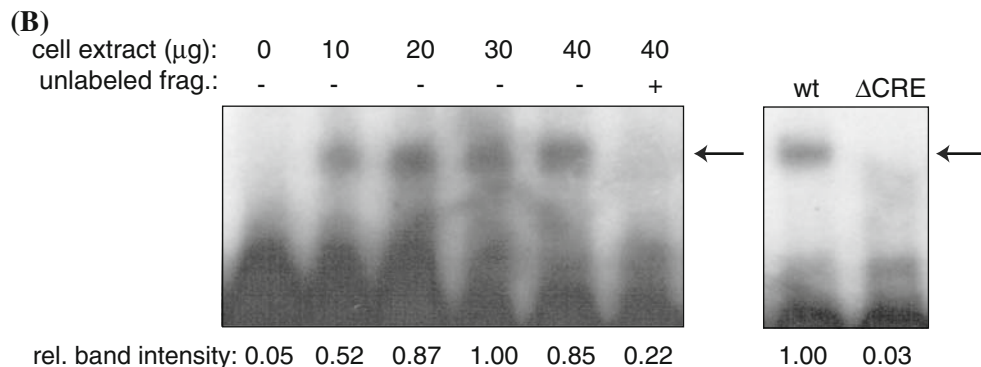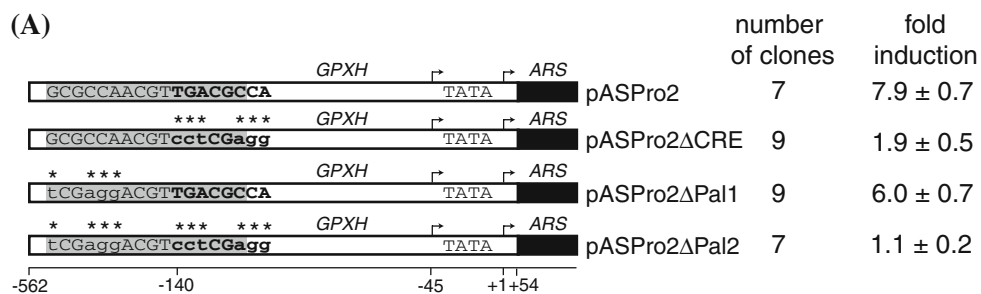To confirm that the CRE/AP-1 element is functioning as a transcription factor binding site, electrophoretic mobility shift assays (EMSA) were performed. A radiolabeled 50 bp DNA fragment of the *GPXH* promoter including the CRE/AP-1 element was incubated with an increasing amount of crude cell extract isolated from a *C. reinhardtii* culture and subsequently separated on a 4% polyacrylamide gel. A retarded band was observed on the gel indicating the formation of a protein complex bound to the labeled DNA fragments (Fig. 3b). The intensity of the bands increased with increasing amounts of crude extract reaching a maximum at 30 μg of extract. The labeled DNA fragments could be competed out by adding an excess of unlabeled fragments showing the formation of a specific DNA binding complex. However, when the same DNA fragment without a functional CRE/AP-1 motif was used, no shifted band was detected.

The CRE/AP-1 motif was further characterized and compared to the two known *cis*-regulatory elements CRE and AP-1 by introducing specific site-directed mutations in various positions of the element. A change of the last base of the element from A to T or of base four from C to G alters the homology to the CRE (TGACGTCA) (Deutsch et al. 1988) but not to the AP-1 (TGA$^C/_G$TCA) consensus sequence (Angel et al. 1987). These mutations resulted in a similar threefold to fourfold reduction in the response to NR (Fig. 3c). However, when the sixth base of the element was changed from C to T to generate a full CRE consensus sequence, the induction was still reduced by twofold. Finally, a full AP-1 consensus sequence (TGACTCA) was introduced in the position of the CRE/AP-1 element and tested for the response to NR. These base changes reduced NR response to an average induction of 2.6-fold.

An alternative way to test for a functional CRE was chosen by analyzing the response of the gene to increased cellular levels of cyclic nucleotides. Therefore, cultures were exposed to the cell-permeable cyclic nucleotide analogues 8Br-cAMP and 8Br-cGMP in the presence or absence of 2 μM NR and tested for upregulation of the wild-type *GPXH-ARS* reporter construct. Surprisingly, both cyclic nucleotide analogues inhibited the response of the reporter construct to NR but did not increase basal expression (Fig. 3d). MDL-12, on the other hand, an inhibitor of the cyclases responsible for cyclic nucleotide synthesis, stimulated pASPro2 expression in both control and NR-treated cultures.

Identification of *cis*-regulatory elements in the *GPXH* promoter

Identification of the CRE/AP-1 motif as functional regulatory element raised the question whether this element is sufficient to stimulate the expression of genes by $^1O_2$. To answer this question, the *GPXH* CRE/AP-1 element was introduced into the β_2-tubulin (*TUBB2*) promoter at the same position as found in the *GPXH* promoter. This resulted in the

(A)

	number of clones	fold induction
pASPro2	7	7.9 ± 0.7
pASPro2ΔCRE	9	1.9 ± 0.5
pASPro2ΔPal1	9	6.0 ± 0.7
pASPro2ΔPal2	7	1.1 ± 0.2

(B)

(C)

	number of clones	fold induction
pASPro2	7	7.9 ± 0.7
A → T	9	2.3 ± 0.4
C → G	9	2.0 ± 0.5
C → T (CRE)	11	4.0 ± 0.3
G → T (AP-1) C → A	14	2.6 ± 0.3

(D)

constructs pYSn2, with only the CRE/AP-1 core sequence, and pYSn3, with the core element and a six nucleotide flanking sequence in the *TUBB2* promoter (Fig. 4a). Construct pYSn4, with only the *TUBB2* promoter, and pYSn1, with a 194 bp *GPXH* promoter fragment (just upstream of the CRE/AP-1 element), were included as controls. No or only a very low induction could be detected with all the *TUBB2* constructs even when they contained the CRE/AP-1 element. On the other hand, average stimulation of pYSn1 expression by NR still reached wild-type levels, even though there was an unusual high variability in the response of clones with this construct (0.9- to 30-fold induction).

◀ **Fig. 3 a** Different *GPXH-ARS* reporter constructs were cloned to analyze the role of a 16 bp palindrome (*grey bar*) and the overlapping CRE/AP1 homologous sequence element (TGACGCCA, *bold letters*) in the response to $^1O_2$. Construct pASPro2ΔCRE has a mutated CRE/AP1 element, pASPro2ΔPal1 was changed in the 5′ part of the 16 bp palindrome and in pASPro2ΔPal2 the whole palindrome was replaced (mutated bases are in *lower case letters* and marked by *stars*). Induction by 2 μM NR was analyzed in the indicated number of independent transformants (average ± SE). **b** Electrophoretic Mobility Shift Assays were performed with crude cell extracts from *C. reinhardtii* and a radioactively labeled DNA fragment of the *GPXH* promoter as template (see Supplementary Table 1). Increasing amounts of cell extract were incubated with labeled DNA fragments in the presence or absence of a 20-fold excess of the same unlabeled DNA fragment and separated on a 4% polyacrylamide gel. Similarly, 30 μg of cell extract were incubated with the same fragment lacking the CRE/AP1 element (ΔCRE) and analyzed on a gel. Intensity of the retarded bands (*arrows*) was quantified with ImageJ (RSB at NIH) and normalized to the most intense band. **c** Various point mutations were introduced into the CRE/AP1 element of the pASPro2 reporter construct (*bold letters*) and their effects on the induction by 2 μM NR were tested in the indicated number of independent transformants. The bases exchanged, their positions in the element (*lower case letters* and *stars*) and the introduction of consensus sequences are indicated and were chosen to change homology to a CRE or AP-1 element (see text for detailed description). **d** Effect of the cyclic nucleotide analogues 8Br-cAMP and 8Br-cGMP or the adenylate cyclase inhibitor MDL-12 on the expression of the pASPro2 reporter construct was tested in control (*white bars*) or 2 μM NR-treated cultures (*black bars*)

Contrary to *GPXH*, the *TUBB2* promoter does not contain a consensus TATA box which might be essential to efficiently induce the expression. Indeed, a *GPXH* reporter construct with a mutated TATA box (pASPro2ΔTATA) showed no induction by the production of $^1O_2$ anymore (Fig. 4a). To test the requirement of a consensus TATA box for $^1O_2$ response, the 3′ part of the *TUBB2* promoter up to position −32 in above constructs (pYS2 to pYS4) was again exchanged by the same size *GPXH* core promoter containing a TATA box (pYS5 to pYS7). Still, induction of these constructs was very low. This indicates that the CRE/AP-1 element and a consensus TATA box are not sufficient to mediate the response to $^1O_2$ and other elements seem to be involved in the process.

Three additional putative *cis*-elements have been found in the *GPXH* promoter by homology to known transcription factor binding site (Leisinger et al. 1999). This includes a putative GC box and two CAAT boxes, one of which is located on the antisense strand (ATTG box). All three elements were mutated individually in a *GPXH-ARS* reporter construct resulting in plasmid pASPro2ΔGC, pASPro2ΔCAAT and pASPro2ΔATTG (Fig. 4b). Two of these constructs (pASPro2ΔGC and pASPro2ΔATTG) exhibited no induction by NR anymore, whereas a mutation of the putative CAAT box on the sense strand did not affect the response of the promoter to NR.

Because the *GPXH* gene gives rise to two independent transcripts we wanted to know which transcript was measured with the reporter constructs. Sequence analysis showed that an artificial stop codon was introduced into the coding sequence of the longer transcript during the cloning process (data not shown). Thus, arylsulfatase activity of clones with the reporter constructs only represents the expression of the shorter transcript. To investigate the role of the identified regulatory elements in the expression of individual transcripts, expression profiles of both transcripts of the reporter gene were investigated by qPCR in one representative transformant. Comparable induction profiles were obtained for the reporter construct pASPro1, containing the wild-type *GPXH* promoter, and the endogenous *GPXH* gene under all conditions tested (Figs. 1, 5). Mutating the 5′-part of the palindrome in pASPro2ΔPal1 did not strongly affect the response of any of the transcripts to RB and high light treatment. In the strains containing the plasmids pASPro2ΔCRE, pASPro2ΔGC or pAS-Pro2ΔATTG, no stimulated response of either transcript could be detected for any of the stress conditions tested. Furthermore, the expression of the longer transcripts of constructs pASPro2ΔGC and pASPro2ΔATTG was strongly reduced under all conditions close to the detection limit. In the strain containing pASPro2ΔTATA, on the other hand, the response of the long transcript to the different stresses was not affected, whereas the shorter transcript had a reduced basal expression and a lower induction under stress conditions.

Discussion

The *GPXH* gene seems to encode a plastidic and cytosolic thioredoxin peroxidase

Similar to GPXs from higher plants (Herbette et al. 2002; Iqbal et al. 2006; Jung et al. 2002; Navrot et al. 2006), GPXH uses TRX to reduce different peroxides like H_2O_2, t-BOOH and CUOOH (Table 1; Fig. 2). This supports the hypothesis that all plant NS-GPX isoforms are actually TRX-dependent peroxidases and even might be a novel class of peroxiredoxins (Herbette et al. 2007; Rouhier and Jacquot 2005; Tanaka et al. 2005). Interestingly, NTRC, a protein containing both an NADP-thioredoxin reductase (NTR) and a TRX domain in a single polypeptide chain (Serrato et al. 2004), was recently shown to function as an efficient electron donor to 2-cys peroxiredoxins (Kirchsteiger et al. 2009; Pérez-Ruiz et al. 2006). Thus, it will be interesting to determine in future studies the role of NTRC, which might also play a role in the reduction of GPXH. Consistent with peroxiredoxins, a conserved resolving cysteine involved in the regeneration of NS-GPX by TRX was also identified in
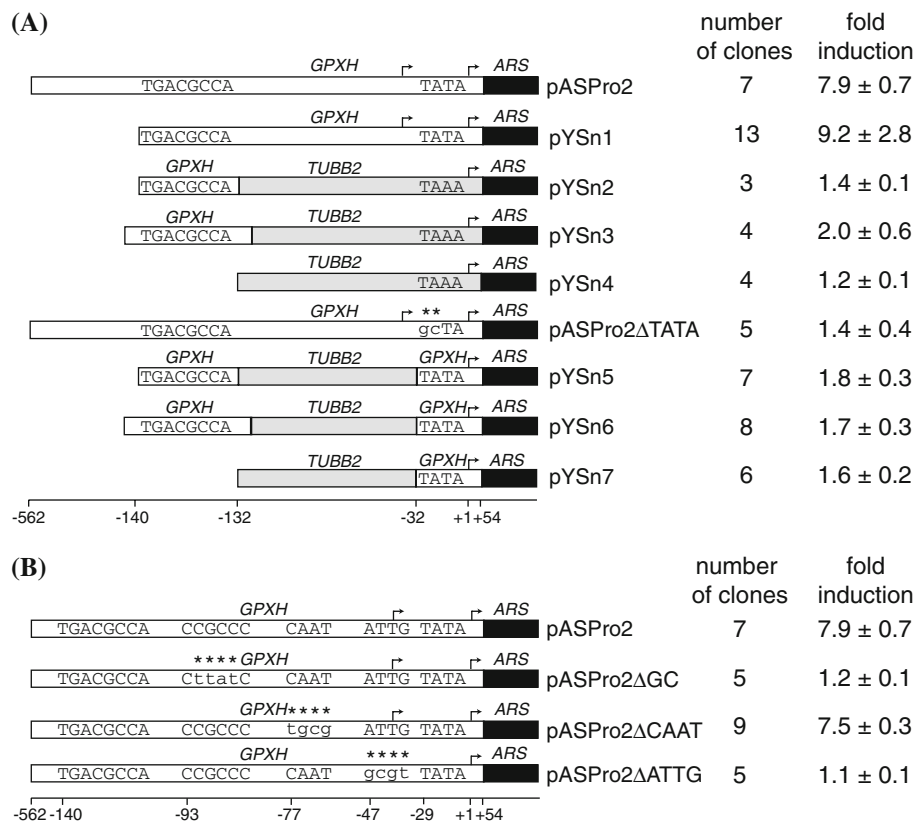
(A)

	number of clones	fold induction
GPXH — TGACGCCA — TATA — ARS — pASPro2	7	7.9 ± 0.7
GPXH — TGACGCCA — TATA — ARS — pYSn1	13	9.2 ± 2.8
GPXH — TUBB2 — TGACGCCA — TAAA — ARS — pYSn2	3	1.4 ± 0.1
GPXH — TUBB2 — TGACGCCA — TAAA — ARS — pYSn3	4	2.0 ± 0.6
TUBB2 — TAAA — ARS — pYSn4	4	1.2 ± 0.1
GPXH — TGACGCCA — ** gcTA — ARS — pASPro2ΔTATA	5	1.4 ± 0.4
GPXH — TUBB2 — GPXH TGACGCCA — TATA — ARS — pYSn5	7	1.8 ± 0.3
GPXH — TUBB2 — GPXH TGACGCCA — TATA — ARS — pYSn6	8	1.7 ± 0.3
TUBB2 — GPXH TATA — ARS — pYSn7	6	1.6 ± 0.2

-562   -140   -132   -32   +1 +54

(B)

	number of clones	fold induction
GPXH — TGACGCCA — CCGCCC — CAAT — ATTG TATA — ARS — pASPro2	7	7.9 ± 0.7
**** GPXH — TGACGCCA — CttatC — CAAT — ATTG TATA — ARS — pASPro2ΔGC	5	1.2 ± 0.1
GPXH **** — TGACGCCA — CCGCCC — tgcg — ATTG TATA — ARS — pASPro2ΔCAAT	9	7.5 ± 0.3
GPXH — **** — TGACGCCA — CCGCCC — CAAT — gcgt TATA — ARS — pASPro2ΔATTG	5	1.1 ± 0.1

-562 -140   -93   -77   -47   -29   +1 +54

Fig. 4 a Reporter constructs with different fusions of either the *GPXH* or *TUBB2* promoter (pYSn2 to pYSn4) to the *ARS* reporter gene. pYSn1 contains a 194 bp short *GPXH* promoter fragment. pYSn2 to pYSn4 consist of a fusion of either a 20 bp (pYSn3), a 8 bp (pYSn2) or no (pYSn4) DNA fragment of the *GPXH* CRE/AP1 element to the *TUBB2* promoter. pASPro2ΔTATA has a *GPXH* promoter with a mutated TATA box (mutated bases are in *lower case letters* and marked by *stars*). In constructs pYSn5 to pYSn7, finally, a 86 bp fragment of the *TUBB2* promoter's 3′ part in pYSn2 to pYSn4 was replaced by a 86 bp *GPXH* promoter fragment including the TATA box. **b** The effect of mutations in either of three putative regulatory elements was analyzed in different *GPXH-ARS* reporter constructs. Elements were identified by their homology to a GC-box/Sp1-element (CCGCCC) or a CAAT-box on the sense (CAAT) or the antisense strand (ATTG). Mutated bases are in *lower case letters* and marked by *stars* and other promoter elements are as described earlier. Induction by 2 μM NR was analyzed in the indicated number of independent transformants (average ± SE)

GPXH (Dayer et al. 2008) and GPXH was retained on a cytosolic TRXh1 affinity column loaded with *Chlamydomonas* soluble extracts (Lemaire et al. 2004). Furthermore, *TRXh1* expression was induced under similar oxidative stress conditions like *GPXH* (Fischer et al. 2005; Ledford et al. 2007). Other reductants like GSH and NADPH and even some chloroplastic TRXs did not support GPXH peroxidase activity indicating a strong specificity of the enzyme for its electron donor. Substrate specificity, on the other hand, was much broader allowing the reduction of H_2O_2 and organic hydroperoxides at similar rates. The activity of GPXH is in the range of other TRX-dependent peroxidases and both catalytic efficiency and substrate specificity are very similar to GPXs of poplar (Navrot et al. 2006) showing that GPXH is a TRX-dependent peroxidase similar to NS-GPX found in higher plants.

The specificity of GPXH for its reductants was identical to poplar chloroplastic GPXs which can use TRXh and TRXy as electron donor whereas the cytosolic GPX isoforms only reacted with TRXh (Fig. 1; Navrot et al. 2006). This strongly supports an activity and thus a function of GPXH in the chloroplast. This would also be consistent with the increased expression of *GPXH* under high light illumination which stimulates the production of ROS and lipid peroxidation in the plastid (Fischer et al. 2006). So far there was no evidence for a targeting of GPXH to the chloroplast. However, data from a *C. reinhardtii* EST library and gene expression analysis (Fig. 1c) revealed that the *GPXH* gene gives rise to two independent transcripts which probably encode a dual-targeted protein, one form targeted to the cytoplasm and the other to the chloroplast (Dayer et al. 2008). In Arabidopsis, as many as 49 dual-targeted proteins were identified so far (Carrie et al. 2009) including the GSTF8 gene, where two independent transcription and translation start sites give rise to a plastid and cytosol-localized protein (Thatcher et al. 2007). A third example for this mechanism might be a putative short chain dehydrogenase (EST clone 984028F11) from *C. reinhardtii*
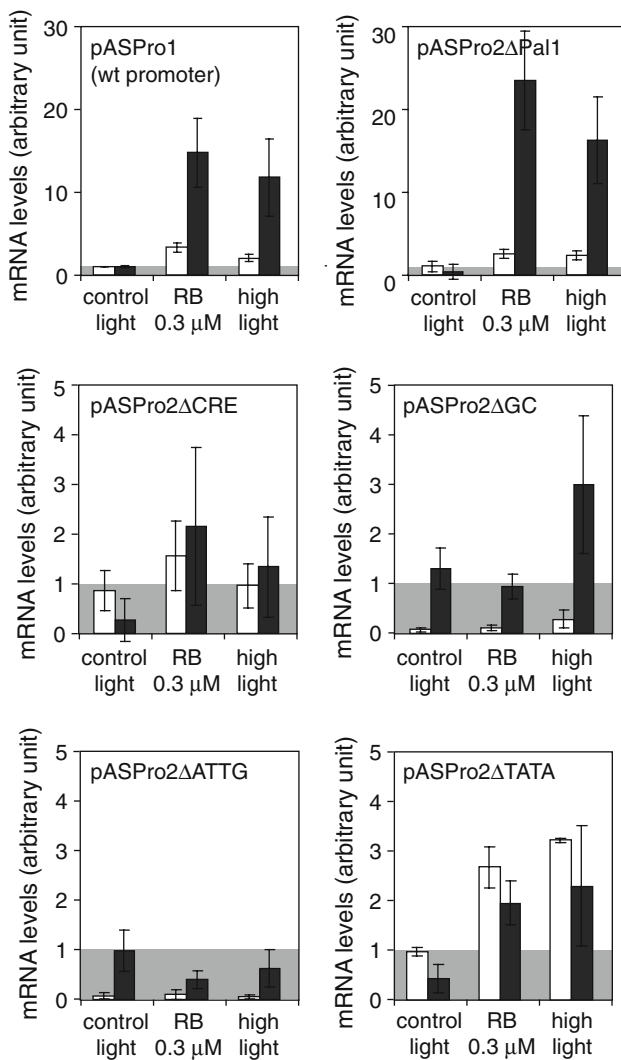
Fig. 5 The expression of the long (*white bars*) and the short transcripts (*black bars*) of various *GPXH-ARS* reporter constructs described in Figs. 3 and 4 was measured in cultures exposed to the stress conditions indicated for 1 h. The expression of individual mRNA (average ± SE) was normalized to the level of the long transcript of pASPro1 (*grey background*)

induced by NR (Fischer et al. 2005), suggesting that the use of different transcription and translation start sites is a prevalent mechanism to direct the same protein to different cellular compartments.

Still, it is an open question why the expression of the cytosolic *GPXH*short is much stronger induced during high light stress than the plastid-directed *GPXH*long, at least at the mRNA level. It appears that GPXH might be involved in the defense against oxidative stress also in the cytoplasm during plastid-derived photooxidative stress. H_2O_2 can easily diffuse across membranes reaching different cellular compartments and $^1O_2$ produced in overexcited PSII reaction centers of *Chlamydomonas* was shown to diffuse to the cytoplasm (Fischer et al. 2007b). Support for an

anti-oxidative function of GPXs in the cytoplasm comes from experiments showing that overexpression of a *Chlamydomonas* GPX-like protein in both the chloroplast or the cytoplasm of tobacco plant strongly increased the resistance to oxidative stress (Yoshimura et al. 2004). All these data support an important role of GPXH as TRX-dependent peroxidase functioning in the chloroplast and cytoplasm of photooxidatively stressed *C. reinhardtii* cells.

Induction of the *GPXH* gene by singlet oxygen required the combined action of multiple regulatory and core promoter elements

Originally, five different regulatory elements and one core promoter element (TATA-box) were identified in the *GPXH* promoter by sequence homology (Leisinger et al. 1999). Removing the TATA-box from the *GPXH* promoter strongly reduced the basal expression and the induction of *GPXH*short but not of *GPXH*long (Fig. 5) suggesting that the TATA box is the core element of the downstream *GPXH*short promoter. At the transcription start site of *GPXH*long, a sequence element (CTATTGA) with similarity to the consensus sequence of the Inr element (PyPyAN-$^T/_A$PyPy), another important core promoter element (Juven-Gershon et al. 2008; Smale and Kadonaga 2003; Yamamoto et al. 2007), and to the transcription start site-motif of TATA-less plant promoters was identified (Shahmuradov et al. 2003). This Inr element was removed from the *GPXH* promoter in construct pASPro2ΔATTG where the overlapping ATTG element was mutated. This reduced the expression of *GPXH*long to <9% of the expression of *GPXH*short (Fig. 5) presumably due to the absence of transcription initiation by the Inr element. In plants, it was found that most photosynthetic nuclear genes lack a TATA box, and that an Inr element was required for proper regulation of the tobacco *psaDb* gene by light (Nakamura et al. 2002). In *Chlamydomonas*, we identified putative Inr elements in the *GSTS1* promoter (CCAGACT) and the downstream promoter of the *HSP70A* gene (TCAACTC) (data not shown), even though the latter also contains a putative TATA-box (Kropat et al. 1995), suggesting that Inr-elements are common in algae too. Furthermore, mutating the Inr element, presumably functioning as core promoter element for *GPXH*long, also abolished the induction of *GPXH*short. Thus, there is either an interaction of the Inr element directly or of the CAAT box overlapping the Inr element with the response of *GPXH*short. Positive interaction between TATA-box and Inr element-driven gene expression has been described before (Emami et al. 1997). However, since in that study the Inr element and the TATA box were part of the same promoter, it is not clear if in *GPXH* the Inr element can affect directly the induction of *GPXH*short because the element is part of a different promoter in the gene. Alternatively, the loss of binding of

transcription factors to the mutated Inr element could alter chromatin structure and thereby reduce the binding of other transcriptional activators to the promoter. A similar effect was suggested for a mutation of the TATA-box on HSP70A-mediated transgene activation (Lodha et al. 2008).

The consensus sequence of a CAAT box is CCAAT which is not 100% identical to the two elements found in *GPXH*, even though some heterogeneity for the first base was reported in plants (Shahmuradov et al. 2003). Still, no clear function of the two putative CAAT boxes in the *GPXH* promoter could be shown. Whereas mutations in the downstream element on the antisense strand (ATTG) interact with the transcription initiation site (Inr element) of *GPXH*long, removing the upstream CAAT box did not affect the expression of the reporter construct (Fig. 4b). The GC-box and the CRE/AP-1 binding site, on the other hand, were both shown to be required for *GPXH* induction. The GC-box is identical to the binding site of the mammalian Sp1 transcription factor (GGGCGG), which is activated by phosphorylation and can be stimulated by various signals including viral infection, growth factors or mechanical stress (Chu and Ferro 2005). In plants, no Sp1 binding sites could be identified so far indicating that they might be absent in these organisms (Yamamoto et al. 2007). In *C. reinhardtii*, mutating the GC-box in the *GPXH* promoter abolished *GPXH*long expression and strongly reduced *GPXH*short induction similar to the mutation in the ATTG (Inr) element. The reduced basal expression of *GPXH*long suggests that there might be an interaction between the GC-box (Sp1 site) and the Inr element. A strong preference for interaction with Inr elements has been previously observed for Sp1 (Emami et al. 1995). The cooperative binding of transcription factors to these elements might be responsible for *GPXH*long expression whereas the basal expression of *GPXH*short seems not to be dependent on this interaction (Fig. 5). Thus, the GC-box might also be part of the *GPXH*long core promoter. Whether the loss of the stress-induced induction of *GPXH*short in pASPro2ΔGC is directly caused by the missing GC-box as a regulatory element or indirectly by the absent *GPXH*long basal expression is not clear. However, the second possibility is consistent with the effect found for the mutated Inr element (Fig. 5) and would suggest that *GPXH*short induction is dependent on the functional expression of *GPXH*long.

The CRE/AP-1 element was identified as regulatory element in *GPXH* before and was thus studied in more details (Leisinger et al. 2001). Electrophoretic mobility shift assays proved its function as a specific transcription factor binding site which is lost by mutating the 8 bp element (Fig. 3b). Furthermore, a role of a 16 bp palindrome overlapping the CRE/AP-1 binding site in the response to $^1O_2$ could be excluded by mutating the 5′ part of the

palindrome (Fig. 3a). Still, the 8 bp CRE/AP-1 core element either with or without flanking sequence was not sufficient to mediate $^1O_2$ response to a recombinant *TUBB2* promoter or a minimal *GPXH*short core promoter (Fig. 4a). Since both elements required for *GPXH*long basal expression (GC-box and Inr element) were also found to be essential for *GPXH*short induction, it could be that these two or even more elements are the missing factors to construct a $^1O_2$ responsive promoter. It seems that the induction by $^1O_2$ is mediated by the combined action of multiple regulatory mechanisms involving different transcription factor binding sites what would be an elegant way to control the response of few genes to one specific signal.

Attempts to identify the CRE/AP-1 site as either a true CRE or AP-1 element failed because introduction of either of two consensus sequences of these elements into the reporter construct resulted in lower induction than with the *GPXH* wild-type element (Fig. 3c; Angel et al. 1987; Deutsch et al. 1988). Further evidence against an induction by a cAMP signaling pathway comes from the inhibition of *GPXH* expression by the cyclic nucleotide analogues 8Br-cAMP and 8Br-cGMP and the stimulation by MDL-12, an inhibitor of adenylate cyclases (Fig. 3d). In humans, both stimulating and inhibiting effects of CRE-like elements in the same promoter have been observed for the expression of the 5-aminolaevulinate synthase gene (Giono et al. 2001). An important role for the opposite responses of this gene has been ascribed to the CREB binding protein CBP, which as a limiting factor for activation might be sequestered away from one transcriptional complex to another by the activation of an alternative signaling pathway (Guberman et al. 2003). Another example for both positive and negative regulation of gene expression through a CRE element was found in a human aromatase gene where the binding of CREB and a member of the Jun family to the same promoter element causes opposite responses (Ghosh et al. 2005). Finally, cAMP could modify the nuclear localization of a *GPXH*-activating transcription factor as described for the stress regulated transcription factor Msn2 in yeast, where high levels of cAMP inhibit the accumulation and thus the activity of the protein in the nucleus (Gorner et al. 2002).

Another regulatory element containing a TGACG motif is the as-1 element found in some auxin and salicylic acid-induced genes including several glutathione-*S*-transferases (GST) of tobacco and Arabidopsis (Chen et al. 1996; Droog et al. 1995; Rodriguez Milla et al. 2003; Ulmasov et al. 1994; van der Zaal et al. 1996). The consensus sequence of an as-1 element consists of the same palindrome as the CRE element (TGACGTCA) but in two copies spaced by four nucleotides (Ellis et al. 1993; Lam et al. 1989). However, only one copy of a TGACG element is present in *GPXH* indicating that it is also different from the as-1 element. Still, in tobacco the as-1 element was

identified as an oxidative stress responsive element and the activation by salicylic acid was found to be mediated by ROS (Garreton et al. 2002). The same element was suggested to be responsible for induction of an *Arabidopsis* phospholipase A gene by paraquat and RB (Narusaka et al. 2003). The as-1 element is recognized by a basic/leucine zipper transcription factor of the TGA-family which was shown to interact with glutaredoxin in Arabidopsis indicating a redox activation by this protein (Ndamukong et al. 2007). Together with the fact that the core of the antioxidant responsive element (ARE) also contains a TGAC motif (Wasserman and Fahl 1997) and that AP-1 can be activated by oxidative stress (Karin et al. 2001), there seems to be a common link between oxidative stress response and TGAC promoter elements. Flanking sequences and adjacent elements might further specify the affinity for individual transcriptional activation complexes (Wasserman and Fahl 1997) and only the identification of the active DNA binding complex might reveal some of the components of the $^1O_2$ signal transduction pathway in *C. reinhardtii*.

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