Identification and functional analysis of peroxiredoxin isoforms in Euglena gracilis

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Published online: 21 May 2014.
Identification and functional analysis of peroxiredoxin isoforms in *Euglena gracilis*

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Received October 17, 2013; accepted December 2, 2013

http://dx.doi.org/10.1080/09168451.2014.890037

*Euglena gracilis* lacks catalase and contains ascorbate peroxidase (APX) which is localized exclusively in the cytosol. Other enzymes that scavenge reactive oxygen species (ROS) in *Euglena* have not yet been identified; therefore, ROS metabolism, especially in organelles, remains unclear in *Euglena*. The full-length cDNAs of four *Euglena* peroxiredoxins (*EgPrxs*) were isolated in this study. *EgPrx1* and -4 were predicted to be localized in the cytosol, and *EgPrx2* and -3 in plastids and mitochondria, respectively. The catalytic efficiencies of recombinant *EgPrx* were similar to those of plant thiol-peroxidases, but were markedly lower than those of APX from *Euglena*. However, transcript levels of *EgPrx1*, -2, and -3 were markedly higher than those of APX. The growth rate of *Euglena* cells, in which the expression of *EgPrx1* and -4 was suppressed by gene silencing, was markedly reduced under normal conditions, indicating physiological significance of Prx proteins.

Key words: peroxiredoxin; *Euglena gracilis*; reactive oxygen species

Reactive oxygen species (ROS) including superoxide, H₂O₂, and other free radical molecules are known to be the by-products of aerobic metabolism and cause oxidative damage to cells. In addition to this cytotoxic effect, ROS are important second messengers that regulate growth, development, and stress responses in various organisms.¹⁻³ Therefore, intracellular levels of ROS concentrations must be tightly controlled. Photosynthetic organisms have developed various non-enzymatic and enzymatic systems to withstand oxidative damage and/or modulate ROS-induced oxidative signaling. The main ROS-scavenging enzymes of plants include superoxide dismutase, ascorbate peroxidase (APX), catalase, and peroxiredoxin (Prx). Even though plants have orthologous genes for glutathione peroxidase (GPX), these have been classified as a novel clade of Prx family because of their donor specificity.⁴⁻⁵ These enzymes have been identified in almost every subcellular compartment in higher plants, including the cytosol, chloroplasts, mitochondria, and peroxisomes.⁶ These ROS-scavenging pathways have been shown to modulate the steady-state levels of ROS in different cellular compartments for signaling purposes as well as protection against oxidative damage. The ROS-scavenging system of a photosynthetic pro-tist, *Euglena gracilis*, has been shown to differ from that of plants.⁷ *Euglena* lacks catalase, and contains a single APX, which is localized exclusively in the cytosol, but not in any other organelle.⁸ Unlike APxs in higher plants, APX in *Euglena* consists of two entirely homologous catalytic domains, forms an intramolecular dimeric structure, and can reduce alkyl hydroperoxides as well as H₂O₂.⁹ We previously demonstrated that silencing APX expression in *Euglena* cells resulted in a significant increase in cellular H₂O₂ levels, which indicated its physiological importance in the regulation of H₂O₂ levels. Enzymatic activities that comprise the ascorbate (AsA)-glutathione (GSH) cycle, including monodehydroascorbate reductase, dehydroascorbate red uctase, and GSH reductase, have also only been found in the cytosol.¹⁰ Although *Euglena* contains non-selenium GPX, which uses reduced GSH as an electron donor,¹¹ the gene encoding GPX has not yet been identified. Therefore, because the identities of other ROS-metabolic enzymes have not yet been determined, ROS metabolism in *Euglena* cells, especially in its organelles, remains unclear.

Prxs constitute a family of thiol-based peroxidases found in all biological kingdoms from bacteria to plants and animals. Prxs have been classified into four types based on their structural and catalytic properties: 1-Cys-Prx, 2-CysPrx, type II Prx (PrxII), and PrxQ.¹² 2-CysPrx, PrxII, and PrxQ contain two catalytic Cys residues, which are essential for the reduction of peroxides. During the catalytic cycle, one Cys residue is oxidized to sulfenic acid, whereas H₂O₂ and alkyl hydroperoxides are reduced to water or their corresponding alcohols.

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Abbreviations: AsA, ascorbate; APX, ascorbate peroxidase; dsRNA, double-stranded RNA; GSH, glutathione; GPX, glutathione peroxidase; KD, knock down; NTR, NADPH-dependent thioredoxin reductase; Prx, peroxiredoxin; RNAi, RNA interference; ROS, reactive oxygen species; TMHMM, transmembrane helices based on a hidden Markov model; Trx, thioredoxin.

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The oxidized Cys residue forms a disulfide bridge with the other Cys residue, which is then resolved by thioredoxin (Trx).

As for physiological importance of ROS-metabolic enzymes in higher plants, it has genetically revealed by several research groups. Tobacco plants overexpressing thylakoid membrane-bound APX (iAPX) showed increased tolerance to oxidative stress and chilling stress under high-light condition. Furthermore, it has revealed by a conditional expression system for Arabidopsis tAPX that the enzyme plays a key role in controlling some gene expression levels in response to cellular redox status. In addition to APX, Arabidopsis mutants deficient in 2-CysPrx had significantly higher H2O2 levels in their leaves, which supported the protective role of Prx in oxidative stress. Arabidopsis mutants deficient in one of the GPX isoforms, AtGPX3 and AtGPX8, were shown to be sensitive to oxidative stress. These findings suggest that Prxs and GPxs are important components of ROS scavenging in photosynthetic organisms as well as APxs. Furthermore, Prxs were shown to not only modulate cellular ROS-dependent signaling, but also, depending on the Prx type, sense the redox state, transmit redox information to binding partners, and function as a chaperone. The multiple functions of Prxs support their physiological importance in redox regulation and the stress response.

We recently performed RNA-seq analysis and identified a series of Euglena full-length cDNA sequences (Ishikawa et al., unpublished data). Although genetic information on ROS metabolic enzymes in Euglena has been limited, except for APX, at least four genes coding putative Prx proteins (EgPrx1-R, 5′-AACTGCAGCTGTATGGGAACACCG-3′; Prx1-F, 5′-TTTCTCGAGCCACGCAACTGTCAGCATCC-3′; Prx2-R, 5′-GAAGGCCTAGGCTCCCACTT AGATTGCC-3′; Prx3-F, 5′-CATATGTCACAAAGGGGCTTCGTTCC-3′; Prx3-R, 5′-GAATCTCAATATTGCTTTAGGGCTTTG-3′; Prx4-F, 5′-CATATGCCGTGCGTGA-CACC-3′; Prx4-R, 5′-GGATCTTACCCTTCGAAGGAAAGGC-3′). The amplified fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI) for sequencing to confirm the absence of PCR errors. The plasmids were digested with XhoI and PstI for Prx1, SacI and Hind III for Prx2, NdeI and BamHI for Prx3 and Prx4, and the resulting DNA fragments were ligated into a pcDNA II vector (Takara, Shiga, Japan) to produce His6-tagged recombinant proteins. The Escherichia coli strain BL21 Star (Agilent Technologies, Santa Clara, CA) was used as a host for the expression of recombinant proteins. Full-length cDNA encoding Trx (accession number YGR209C) and NTR (accession number YDR353 W) from yeast was amplified by PCR with primers (Trx-F, 5′-GAGCTCATGGTCACTCAATTAAAATC-3′; Trx-R, 5′-GGATCCCTTACCCTTCGAAGGAAAGGC-3′; ScNTR-F, 5′-GAGCTCATGGTCACTCAATTAAAATC-3′; ScNTR-R, 5′-GGATCCCTTACCCTTCGAAGGAAAGGC-3′; ScNTR-R, 5′-GAGCTCATGTCATTTCTGAGGAATGTAAC-3′), and finally subcloned into pcDNA II vector as well as the procedures for EgPrxs. The resulting constructs were transformed into E. coli BL21 Star.

Expression and purification of recombinant proteins. Transformed cells were grown in LB medium supplemented with 50 μg mL−1 ampicillin at 37 °C. When the culture reached an absorbance of 0.4−0.5 at 600 nm, 0.5 mM isopropyl β-D-thiogalactopyranoside was added, and the cells were grown further at 15 °C for 20 h. E. coli cells were resuspended in 20 mM HEPES-NaOH buffer, pH 7.0, and lysed by sonication. His-tagged recombinant proteins were purified on a column packed with TALON Metal Affinity resin (Clontech, Palo Alto, CA). Finally, the purified enzymes were desalting and concentrated using an ultrafiltration membrane (Amicon Ultra-4, Millipore, Billerica, MA), and stored at −20 °C until used.

Crude extract from Euglena cells. Euglena cells were collected by brief centrifugation and suspended in three volumes of an ice-cold buffer (HEPES-NaOH, pH 7.0) and disrupted by sonication. The cell lysate was centrifuged at 40,000 rpm at 4 °C for 30 min and the supernatant was used for the assay of Prx activity. For the assay of APX and GPX activities, buffer was replaced with 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 1 mM AsA and 50 mM potassium phosphate, pH 7.0, respectively.

Enzyme assays. Trx-dependent peroxidase activity was measured as the decrease in absorbance at 340 nm (ε = 6.22 mM−1 cm−1) due to NADPH oxidation according to Kim et al. The reaction mixture contained 0.2 mM NADPH, 6 μM Trx, 0.3 μM Trx reductase, and 0.1 mM H2O2 in 20 mM HEPES-NaOH, pH 7.0, in a final volume of 0.1 mL. The reaction mixture was pre incubated for 1 min and started by the addition of
H₂O₂. NADPH consumption was monitored by the decrease in absorbance at 340 nm for 1 min. AsA-dependent peroxidase activity was measured as the decrease in absorbance at 290 nm (ε = 2.8 mM⁻¹ cm⁻¹) due to AsA oxidation according to Ishikawa et al.⁸ The reaction mixture contained 1 mM EDTA, 0.4 mM AsA, and 0.1 mM H₂O₂ in 50 mM potassium phosphate buffer, pH 7.0, in a final volume of 1 mL. GSH-dependent peroxidase activity was measured as the decrease in absorbance at 340 nm due to NADPH oxidation. The reaction mixture contained 0.2 mM NADPH, 2 mM GSH, 1 U GSH reductase, and 0.1 mM H₂O₂ in 50 mM potassium phosphate buffer, pH 7.0, in a final volume of 1 mL. The activity of each peroxidase with organic peroxides was also assayed using the same reaction mixture; however, H₂O₂ was replaced with 0.1 mM tert-butyl hydroperoxide or 0.1 mM cumene hydroperoxide.

RNA isolation and quantitative PCR analysis. Total RNA was isolated from Euglena gracilis using RNAiso (Takara). Brieﬂy, Euglena cells were homogenized in 1 mL of RNAiso followed by chloroform extraction at 13,000 rpm for 20 min. RNA in the supernatant was precipitated with an equal volume of isopropanol and washed with 70% ethanol. Total RNA was puriﬁed with a NucleoSpin RNA Plant (Takara) according to the manufacturer’s instructions and quantiﬁed with NanoDrop 1000 (Thermo Fisher Scientiﬁc, Waltham, MA). A 500 ng aliquot of puriﬁed RNA was used for cDNA synthesis using PrimeScript RT Master Mix (Takara) according to the manufacturer’s instructions. The synthesized cDNA was then used in real-time PCR with forward and reverse primers speciﬁc for each of the genes analyzed: EF1α-F, 5'-ACAGATTTGGGAAACGGGTACGCG-3′; EF1α-R, 5'-TTCATCAGGACAATCGCAGCA-3′; Prx1-F, 5'-GAAGTGTGTGACCAGCCAAAC-3′; Prx1-R, 5'-AAGCTGTATGGGAACACCC-3′; Prx2-F, 5'-GGGAGCGCTTGTATATC-3′; Prx2-R, 5'-CACAAGAATTCCCTCCTGG-3′; Prx3-F, 5'-CCTGCCAACTGGACACC-3′; Prx3-R, 5'-ACCACCAACAGCTGC-3′; Prx4-F, 5'-TTCAGAGGTAAACCCGG-3′; Prx4-R, 5'-GACCGCACTGCCAT-3′; APX-F, 5'-GCAGGCGCTGTCGCATACG-3′ and APX-R, 5'-AATGTGCGGCCGTTGTTGAG-3′. Real-time PCR was performed with SYBR Premix Ex Taq (Takara) on the Thermal Cycler Dice Real Time System TP850 (Takara).

RNA interference (RNAi) experiments. Silencing of Prx by RNAi was performed as described previously.⁹ An approx. 500-bp partial Euglena Prx cDNA was PCR-ampliﬁed with the addition of the T7 RNA polymerase promoter sequence (underlined in the primer sequences below) at one end. The primers were Prx1/RNAi-F (5'-TAATACGACTCACTATAGGGGATGTCCAAG-3′), Prx1/RNAi-R (5'-TAATACGACTCACTATAGGGGATGTCCAAG-3′), Prx2/RNAi-F (5'-TAATACGACTCACTATAGGGGATGTCCAAG-3′), Prx2/RNAi-R (5'-TAATACGACTCACTATAGGGGATGTCCAAG-3′), Prx3/RNAi-F (5'-TAATACGACTCACTATAGGGGATGTCCAAG-3′), and APX-R, 5'-AATGTGCGGCCGTTGTTGAG-3′). Sense and antisense RNAs were synthesized using the PCR products as templates (MEGAscript RNAi Kit; Life Technologies, Carlsbad, CA). After puriﬁcation of the transcribed RNA with DNase I digestion followed by ethanol precipitation, double-stranded RNA (dsRNA) was produced by annealing equimolar amounts of the sense and antisense RNAs. Euglena cells from two-day-old cultures were collected and resuspended in KH medium. The cell suspension (100 μL; approx. 1 × 10⁷ cells) was transferred to a 0.2-cm-gap cuvette and electrocorotected with 15 μg of Prx-dsRNAs using NEPA21 (NEPA GENE, Chiba, Japan). The cell suspension was diluted with fresh KH medium and cultured at 22 °C for restoration.

Spot assay. The cell growth rates of control and silenced cells were determined with spot assays. Cultures of each cell were spotted onto KH (heterotrophic) and CM (autotrophic) plates. The numbers of cells spotted were 4 × 10⁴ (1), 2 × 10⁴ (1/2), 1 × 10⁴ (1/4), 5 × 10³ (1/8), and 2.5 × 10² (1/16). Cells were grown under continuous illumination (50 μmol m⁻² s⁻¹) at 22 °C for five days and pictures were taken.

Results and discussion

Primary sequence of four Euglena Prxs. We obtained genetic information on four full-length cDNAs encoding Prx by a tblastn search of our RNA-seq data constructed recently (Ishikawa et al., unpublished data). These genes were designated as EgPrx1–4 (accession number AB853312, AB853313, AB853314, and AB853315, respectively). The cDNA sequences of all EgPrxs, except for EgPrx3, contained a spliced leader sequence, a characteristic motif of Euglena transcripts generated by trans-splicing manner at their 5′ end.²¹ indicated that these cDNA sequences were obtained as full-length cDNA. As for EgPrx3, the first ATG codon appeared following predicted 5′ UTR region, indicating that the clone deﬁnitely contained full-length protein coding region.

Homology analysis indicated that EgPrx1, -2, and -3 were highly identical to each other (approx. 57–83% identity), whereas EgPrx4 had low identity (approx. 22–36% identity) with the other EgPrxs (Supplemental Table 1; see Biosci. Biotechnol. Biochem. http://dx.doi.org/10.1080/09168451.2014.890037). These results suggested that EgPrx1, -2, and -3 belonged to the same subgroup, but were distinct from EgPrx4. To reveal the classification of four EgPrxs, the identities of EgPrxs with known Prxs including all Prx subgroups were determined. Prx1, -2, and -3 were highly identical (approx. 48–75%) to 2-CysPrxs from various organisms such as mammals, plants, algae, and trypanosomatids, whereas EgPrx4 was approx. 45–69% identical to PrxII from algae and plants (Supplemental Table 1). In contrast, these Prxs had low identities (approx. 20–30%
identity) to Prxs in the other subclass. Thus, EgPrx1, 2, and 3 may be classified as 2-CysPrx and EgPrx4 as PrxII.

The sequence alignments and phylogenetic tree of EgPrx1, -2, and -3 with the known 2-CysPrxs and EgPrx4 with the known PrxIIs were constructed using the ClustalW program. As shown in Fig. 1, all EgPrxs had two conserved Cys residues, which were essential for the catalytic reaction of 2-CysPrx and PrxII. All EgPrxs additionally contained conserved Pro, Thr, and Arg residues, which were important for catalytic Cys stability or peroxide reduction in the known 2-CysPrxs and PrxIIs. The phylogenetic tree indicated that EgPrx1 and EgPrx3 were very close to the protozoa clade including Trypanosoma brucei and Leishmania major, while EgPrx2 and EgPrx4 were close to plants and the algae clade, such as Synechocystis sp. PCC6803 (Supplemental Fig. 1).

Each amino acid sequence was analyzed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) programs to predict the subcellular localization of EgPrxs. The TMHMM program revealed that two transmembrane domains existed at the N-terminal region of EgPrx2 (Supplemental Fig. 2(B)), corresponding to a membrane domains existed at the N-terminal region of PrxII. The phylogenetic tree indicated that EgPrx1 and EgPrx3 were very close to the protozoa clade including Trypanosoma brucei and Leishmania major, while EgPrx2 and EgPrx4 were close to plants and the algae clade, such as Synechocystis sp. PCC6803 (Supplemental Fig. 1).

To examine the substrate specificity of rEgPrxs, enzyme activities were measured using H$_2$O$_2$, tert-butylhydroperoxide (t-BOOH), and cumene hydroperoxide (cumene-OOH) as substrates. As shown in Table 1, all EgPrxs, except for rPrx4, catalyzed the reduction of all substrates with activities of 0.25–2.11 μmol min$^{-1}$ mg$^{-1}$ protein. rEgPrx4 could reduce H$_2$O$_2$ and t-BOOH, but not cumene-OOH. Therefore, Euglena Prxs could reduce both H$_2$O$_2$ and alkyl hydroperoxide. barley 2-CysPrx reduced t-BOOH and cumene-OOH at almost the same rates as H$_2$O$_2$. Leishmania donovani TXNPx reacted with H$_2$O$_2$, t-BOOH, cumene-OOH, linoic acid hydroperoxide, and phosphatidyl choline hydroperoxide. Therefore, the broad substrate specificities of EgPrxs were consistent with those of the known Prxs.

A kinetic analysis of rEgPrxs was then performed. As shown in Table 2, the $V_{max}$, $K_m$, and $k_{cat}$ values of rEgPrx1 for H$_2$O$_2$ were 3.15 μmol min$^{-1}$ mg$^{-1}$ protein, 39.1 μM, and 1.21 s$^{-1}$. The catalytic efficiency ($k_{cat}/K_m$) of rEgPrx1 for H$_2$O$_2$ was 3.1 × 10$^4$ M$^{-1}$ s$^{-1}$. The kinetic parameters of rEgPrx2 and rEgPrx3 for H$_2$O$_2$ were similar to those of rEgPrx1. Although the $K_m$ value of rEgPrx4 for H$_2$O$_2$ was 10-fold lower than that of the other EgPrx isoforms, the $V_{max}$ and $k_{cat}$ values of rPrx4 were 4 to 6-fold lower than those of the other Prx isoforms, which indicated that their catalytic efficiencies were nearly equal to each other. The kinetic parameters of these enzymes for t-BOOH and cumene-OOH were similar to those of H$_2$O$_2$. Previous studies reported that the catalytic efficiencies of 2-CysPrx from pea and barley were 2.5 × 10$^9$ and 1.1 × 10$^8$ M$^{-1}$ s$^{-1}$, respectively. Plant GPX isoforms were able to reduce H$_2$O$_2$ and alkyl hydroperoxides using Trx, but not GSH. The $k_{cat}/K_m$ values of Arabidopsis GPX1, 2, 5, and 6 for H$_2$O$_2$ were 4.9 × 10$^9$, 4.5 × 10$^8$, 3.1 × 10$^7$, and 6.1 × 10$^5$ M$^{-1}$ s$^{-1}$, respectively. Thus, the catalytic efficiencies of the rEgPrxs were similar to those of the plant Prxs and GPXs, which suggested that Euglena Prxs functioned as a peroxidase as well as plant enzymes. However, the $k_{cat}$ value and catalytic efficiency of Euglena recombinant APX for H$_2$O$_2$ were...
markedly higher than those of rEgPrxs (577 s$^{-1}$ and $1.65 \times 10^7$ M$^{-1}$ s$^{-1}$, respectively). While we were unable to accurately compare the activity of Prx with that of APX because of electron donor differences between the heterologous Trx/NTR system and AsA, APX may be an efficient peroxidase for cytosolic H$_2$O$_2$ metabolism in Euglena cells.

**Expression analysis of EgPrx1 and EgAPX**

The steady-state transcripts of EgPrx1–4 and EgAPX were detected in Euglena cells grown heterotrophically under normal growth conditions. Quantitative PCR analysis revealed that the transcript levels of EgPrx1–4, and -3 were significantly higher than those of EgAPX, whereas those of EgPrx4 were very low (Fig. 2). In general, Prx is known to accumulate to high concentrations. For example, Arabidopsis 2-CysPrxs have been shown to be among the top 20 most abundant stromal proteins. König et al. estimated the stromal 2-CysPrx concentration to be 60 μM. The high transcript levels of

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**Notes:** Comparison of the predicted amino acid sequences of (A) Euglena Prx1, 2, and 3 with 2-CysPrxs from Trypanosoma brucei (TbCTXNPx), Synechocystis sp. PCC6803 (S6803_2CP), Arabidopsis thaliana (At2CPA), and Homo sapiens (HsPrx1) and (B) Euglena Prx4 with PrxII from Synechocystis sp. PCC6803 (S6803_PrxII), Chlamydomonas reinhardtii (CrPrx3), A. thaliana (AtPrxIIE), and H. sapiens (HsPrx5). Letters shown in white on a black background represent conserved catalytic Cys residues. The residues involved in catalytic Cys stability or peroxide reduction are shown in black letters on a gray background. The box shows the common motif LRR found in the presequence of Euglenozoa mitochondrial proteins. Asterisks indicate amino acids conserved in all sequences, and colons and dots indicate amino acids with similar biochemical characteristics.

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**Fig. 1.** Amino acid sequence alignment of Prxs from Euglena and other organisms.
Table 1. Peroxide specificities of recombinant Euglena Prxs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>rEgPrx1</th>
<th>rEgPrx2</th>
<th>rEgPrx3</th>
<th>rEgPrx4</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>2.11 ± 0.05</td>
<td>2.08 ± 0.08</td>
<td>1.49 ± 0.15</td>
<td>0.42 ± 0.13</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>1.66 ± 0.01</td>
<td>2.01 ± 0.03</td>
<td>0.94 ± 0.04</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>Cumene-OOH</td>
<td>0.80 ± 0.13</td>
<td>1.16 ± 0.14</td>
<td>0.25 ± 0.03</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 2. Kinetic parameters of recombinant Euglena Prxs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{max}$ (μmol min⁻¹ mg⁻¹ protein)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rEgPrx1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td>3.15 ± 0.34</td>
<td>39.1 ± 4.1</td>
<td>1.21 ± 0.13</td>
<td>3.1 × 10⁴</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>2.21 ± 0.21</td>
<td>24.5 ± 4.9</td>
<td>0.85 ± 0.08</td>
<td>3.5 × 10⁴</td>
</tr>
<tr>
<td>Cumene-OOH</td>
<td>1.73 ± 0.09</td>
<td>24.6 ± 2.5</td>
<td>0.66 ± 0.03</td>
<td>2.7 × 10⁴</td>
</tr>
<tr>
<td>rEgPrx2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td>3.34 ± 0.51</td>
<td>44.7 ± 3.1</td>
<td>1.28 ± 0.19</td>
<td>2.9 × 10⁴</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>2.92 ± 0.13</td>
<td>38.9 ± 1.8</td>
<td>1.12 ± 0.05</td>
<td>2.9 × 10⁴</td>
</tr>
<tr>
<td>Cumene-OOH</td>
<td>2.71 ± 0.05</td>
<td>37.7 ± 3.0</td>
<td>1.04 ± 0.02</td>
<td>2.8 × 10⁴</td>
</tr>
<tr>
<td>rEgPrx3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td>2.29 ± 0.16</td>
<td>37.8 ± 6.5</td>
<td>0.88 ± 0.06</td>
<td>2.3 × 10⁴</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>2.22 ± 0.11</td>
<td>36.1 ± 2.6</td>
<td>0.85 ± 0.04</td>
<td>2.4 × 10⁴</td>
</tr>
<tr>
<td>Cumene-OOH</td>
<td>1.33 ± 0.08</td>
<td>22.2 ± 3.0</td>
<td>0.51 ± 0.03</td>
<td>2.3 × 10⁴</td>
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<tr>
<td>rEgPrx4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td>0.59 ± 0.04</td>
<td>3.4 ± 0.5</td>
<td>0.22 ± 0.01</td>
<td>6.6 × 10⁴</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>0.77 ± 0.09</td>
<td>12.1 ± 1.4</td>
<td>0.29 ± 0.04</td>
<td>2.4 × 10⁴</td>
</tr>
</tbody>
</table>

Note: The activity assay was performed using concentrations of 2-100 μM of the peroxide substrates. Each rEgPrx was added at a concentration of 1 μM. Values are the mean ± SD (n = 3).

Effect of Prx suppression on cell growth. Euglena cells were previously shown to epigenetically suppress gene expression by the simple introduction of double-stranded RNA (dsRNA) into cells; however, the transformation procedure of Euglena has not yet been established. Therefore, to evaluate the physiological

Fig. 2. Expression of Prx and APX genes in Euglena.

Notes: Total RNA was extracted from 7-day-old Euglena cells grown heterotrophically under normal growth conditions and was converted into first-strand cDNA using an oligo dT primer. Quantitative PCR analysis was performed to determine the expression levels of Prx1, 2, 3, and 4 and APX. Relative amounts were normalized to EF1α mRNA. Values are the mean ± SD (n = 3).

Table 3. Peroxidase activities in extracts from Euglena cells.

<table>
<thead>
<tr>
<th>Donors</th>
<th>Trx (Yeast)</th>
<th>AsA</th>
<th>GSH</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>127 ± 10</td>
<td>831 ± 21</td>
<td>157 ± 6</td>
<td>n.d.</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>117 ± 15</td>
<td>131 ± 22</td>
<td>92 ± 26</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cumene-OOH</td>
<td>68 ± 13</td>
<td>88 ± 17</td>
<td>72 ± 35</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Note: Peroxidase activities were measured using 6 μM Trx and 0.3 μM NTR from yeast for Prx activity, 0.4 mM AsA for APX activity, and 2 mM GSH and 1 U/mL GSH reductase from yeast for GPX activity. Each peroxidase was added at a concentration of 100 μM. Values are the mean ± SD (n = 3). n.d.; not detected.
significance of Prxs in *Euglena* cells, KD-prx1–4 cells were generated by introducing dsRNA, synthesized from part of each *EgPrx* sequence, into *Euglena* cells using electroporation. The suppression of individual *Prx* gene expression levels in each KD-prx cell was confirmed by RT-PCR (Fig. 3(A)). KD-prx2 and KD-prx4 cells showed significant suppression, whereas KD-prx1, KD-prx3, and KD-prx4 cells showed moderate suppression. KD-prx1/4, KD-prx2/3, and KD-prx3/4 cells showed no suppression. (B) and (C) Prx and APX activities in extracts from each control and each KD cell, respectively. Values are the mean ± SD (n = 3). Values with different letters were significantly different according to the t-test (p < 0.05). (D) and (E) Cultures of control and each KD cell were spotted onto KH (heterotrophic) and CM (autotrophic) plates, respectively. The numbers of cells spotted were 4 × 10⁴ (1), 2 × 10⁵ (1/2), 1 × 10⁵ (1/4), 5 × 10⁴ (1/8), and 2.5 × 10³ (1/16). Plates were incubated under normal conditions for 5 d.

Fig. 3. Effect of Prx suppression on *Euglena* cells.

Notes: (A) RT-PCR analysis of *Prx1–4* and *EF1α* (for normalization) mRNA levels using total RNA from *Euglena* cells in which dsRNA was introduced. The control represented cells electroporated without dsRNA. Fragments corresponding to Prxs and EF1-α were amplified by PCR using cDNA preparations as templates. (B) and (C) Prx and APX activities in extracts from each control and each KD cell, respectively. Values are the mean ± SD (n = 3). Values with different letters were significantly different according to the t-test (p < 0.05). (D) and (E) Cultures of control and each KD cell were spotted onto KH (heterotrophic) and CM (autotrophic) plates, respectively. The numbers of cells spotted were 4 × 10⁴ (1), 2 × 10⁵ (1/2), 1 × 10⁵ (1/4), 5 × 10⁴ (1/8), and 2.5 × 10³ (1/16). Plates were incubated under normal conditions for 5 d.
KD-prx4 cells, in particular, exhibited the specific suppression of corresponding Prx gene expression. On the other hand, due to high sequence similarity of Prxl to Prx3, the expression of both Prx3 and Prxl was more likely to be simultaneously suppressed in KD-prx1 and KD-prx3 cells, respectively. Considering the possibility that other Prx genes, except the targeted Prx, might compensate for the suppression effect, double KD-prx cells, including all EgPrx combinations, were generated by the simultaneous introduction of different dsRNAs. Although KD-prx2/4 cells exhibited the specific suppression of Prx gene expression, the introduction of dsRNAs for individual Prxl and Prx3 in other cells affected the expression of Prx3 and Prxl, respectively, as well as single-KD cells (Fig. 3(A)).

Total Prx activity was significantly lower in all cells in which dsRNA was introduced, except KD-prx4, than in control cells that had been electroporated without dsRNA. KD-prx1 cells exhibited the lowest Prx activity among the single-KD cell lines, though the effect of co-suppression of other homologous Prx genes on total Prx activities is needed to consider. This result supports the high transcript levels of the Prxl gene, as shown in Fig. 2. In the case of double-KD cell lines, total Prx activities in KD-prx1/2, KD-prx1/3, and KD-prx1/4 cells were 19, 33, and 42% that of the control cells, respectively (Fig. 3(B)). As we predicted that Prx suppression would result in the upregulation of APX activity, followed by an ROS metabolism imbalance, total APX activities in control and KD-prx cells were also determined. APX activities in KD-prx cells ranged between 70 and 86% that of the control cells (Fig. 3(C)), suggested that the suppression of Prxs did not stimulate APX expression or post-transcriptional regulation.

To determine the effect of Prx suppression on cell growth, spot assays were performed using heterotrophic and autotrophic media. While the growth rates of all cell types of KD-prx1/4 cells, including all EgPrx combinations, were generated in chloroplasts and mitochondria. In contrast to plants, photosynthesis in Euglena is not susceptible to H2O2 due to the resistance of the fructose-1,6-/sedoheptulose-1,7-bisphosphatase, NADP+-glyceraldehyde-3-phosphate dehydrogenase, and ribulose-5-phosphate kinase enzymes of the Calvin cycle to H2O2 up to 1 mM.39) H2O2 generated in both chloroplasts and mitochondria in Euglena cells diffuses into the cytosol, where it is then subsequently decomposed by APX.40)

In contrast to KD-prx1/4 cells, the suppression of putative organelle-localized Prx (EgPrx2 and -3) did not induce a decrease in the cell growth rate. This may have occurred for the following reasons: (i) different unidentified thiol-peroxidases such as GPX may exist in chloroplasts and mitochondria; (ii) resistance of Calvin cycle enzymes to H2O2; and (iii) the diffusion of H2O2 generated in chloroplasts and mitochondria.

Supplemental material

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.890037.

Funding

This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI [grant number 24380186 (T.I.)]; Grant-in-Aid for Scientific Research (B).

References

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