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# A eukaryotic-like sulfiredoxin involved in oxidative stress responses and in the reduction of the sulfinic form of 2-Cys peroxiredoxin in the cyanobacterium *Anabaena* PCC 7120

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## Summary

• The overoxidation of 2-Cys peroxiredoxins (Prxs) into a sulfinic form was thought to be an irreversible protein inactivation process until sulfiredoxins (Srxs) were discovered. These are enzymes occurring among eukaryotes, which are able to reduce sulfinylated Prxs. Although Prxs are present in the three domains of life, their reduction by Srxs has been described only in eukaryotes so far.

• Here it was established that the cyanobacterium *Anabaena* PCC 7120 has a Srx homologue (SrxA), which is able to specifically reduce the sulfinic form of the 2-Cys Prx (PrxA) both *in vivo* and *in vitro*. A mutant lacking the *srxA* gene was found to be more sensitive than the wild type to oxidative stress.

• Sulfiredoxin homologues are restricted to the cyanobacterial and eukaryotic genomes sequenced so far. The present phylogenetic analysis of Srx and 2-Cys Prx sequences showed a pattern of coevolution of the enzyme and its substrate that must have involved an ancient gene transfer between ancestors of Cyanobacteria and Eukaryotes, followed by a more recent transfer from Cyanobacteria to Plantae through the chloroplastic endosymbiosis.

• This is the first functional characterization of a Srx enzyme in a prokaryotic organism.

## Introduction

Peroxiredoxins (Prxs) are ubiquitous thiol-specific peroxidases that catalyse the reduction of $H_2O_2$, alkyl hydroperoxides and peroxynitrite, using thioredoxin and other thiol-containing reducing agents as electron donors (Dietz, 2007; Poole, 2007). The first step in ROS (reactive oxygen species) reduction by Prxs involves an *N*-terminal cysteine residue in which the thiol side-chain is oxidized into sulfenic acid (SOH). In the second step, the sulfenic acid is re-reduced to a thiol before the next catalytic cycle begins. Among Prxs, typical 2-Cys Prxs are obligate homodimers having a well-known action mechanism (Konig *et al.*, 2003; Guimaraes *et al.*, 2005; Hall *et al.*, 2009). In eukaryotes, the sulfenic form of the 2-Cys Prxs can undergo a further oxidation step, yielding a sulfinic acid form (SOOH). The reduction of the sulfinic form and the subsequent recycling of the active form of 2-Cys Prxs are catalysed by a specific reductase named sulfiredoxin (Srx). After being first characterized in yeast (Biteau *et al.*, 2003), Srxs were identified in several eukaryotes, including plants and humans (Chang *et al.*, 2004; Liu *et al.*, 2006; Rey *et al.*, 2007). The mechanism underlying the Srx-catalyzed reduction of overoxidized Prxs has been thoroughly described (Biteau *et al.*, 2003; Jeong *et al.*, 2006; Jonsson *et al.*, 2008a,b): it involves a conserved cysteine residue and requires ATP hydrolysis, $Mg^{2+}$, and a thiol serving as an electron donor. The overoxidation of 2-Cys Prxs and their reduction by sulfiredoxins were assumed for a long time to be eukaryotic features coselected in the course of evolution, giving Prxs a regulatory function in eukaryotes (Biteau *et al.*, 2003; Wood *et al.*, 2003; Jacob *et al.*, 2004; Bozonet *et al.*, 2005; Jonsson & Lowther, 2007). In prokaryotes, Prxs are thought to be required only for ROS detoxification purposes (Wood *et al.*, 2003). It has therefore been assumed that bacteria do not contain any active Srxs (Biteau *et al.*, 2003; Findlay *et al.*, 2005; Jonsson & Lowther, 2007). However, it was recently reported that 2-Cys Prxs from the cyanobacteria *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 are

sensitive to overoxidation (Pascual *et al.*, 2010). In addition, the fact that overoxidation of the *Anabaena* 2-Cys Prx into a SOOH form can be reversed *in vivo* suggests the presence of Srx activity in this bacterium (Pascual *et al.*, 2010). However, to our knowledge, no such enzyme activity has ever been characterized so far in cyanobacteria. The results obtained here show that the cyanobacterium *Anabaena* PCC 7120 actually possesses a Srx enzyme (SrxA) which is homologous to the eukaryotic Srxs, and that it is able to reduce the SOOH of the 2-Cys Prx (PrxA) both *in vivo* and *in vitro*. Our data also show that Srx occurs in most cyanobacteria. Phylogenetic analyses showed that the enzyme (Srx) and its substrate (2-Cys Prx) have coevolved with and that an early horizontal gene transfer (HGT) of both genes must have occurred between the ancestors of present-day eukaryotes and cyanobacteria. This early transfer was followed later by a second HGT from cyanobacteria to the ancestor of the Plantae via the primary chloroplastic endosymbiosis, which explains the close similarities observed between the cyanobacterial and most plant sequences.

## Materials and Methods

### Bacterial strains, plasmids and growth conditions

*Anabaena* sp. PCC 7120 was grown in BG11 medium at 30°C in air under continuous illumination (40 μmol $m^{-2}$ $s^{-1}$) To analyse the effects of high light intensities, *Anabaena* strains were grown under 150 μmol $m^{-2}$ $s^{-1}$. Cyanobacterial growth was monitored by measuring the absorbance at 750 nm (OD 750).

The *srxA* knock-out mutant (strain *ΔsrxA*) was generated by homologous recombination, substituting the spectinomycin cassette for the *Anabaena srxA* gene (asl4146). A PCR fragment containing the spectinomycin resistance conferring cassette flanked by 500 bp upstream and downstream *srxA* was generated using the following primers: *srx*Δ1 primer 5′-TACCTAAAACCACAACCTCTG-3′, *srx*Δ2 primer 5′-ACTCGAGAGGGATCCTCGAAT TCAGGAGTAAACTCAACTAGTTCCTT-3′, *srx*Δ3 primer 5′-GAATTCGAGGATCCCTCT CGAGTGTAGAGAATAGGAAAAGGCAGG-3′ and *srx*Δ4 primer 5′-AAAATAGAACGTG CTGTTCTCA-3′. After a subcloning step in the Bluescript SK vector, this fragment was cloned into the *Sac*I site of the conjugal suicide plasmid pRL271. The recombinant plasmid obtained was transferred to *Anabaena* using the previously described conjugation method (Cai & Wolk, 1990). *Anabaena* recombinant clones were selected on BG11 supplemented with spectinomycin at 20 μg $ml^{-1}$. Double recombinants were selected on the basis of their ability to grow in the presence of sucrose. Complete segregation of the mutation was confirmed by PCR. Three independent conjugations were performed and clones obtained from each experiment were used in all subsequent studies on the mutant.

### Expression and purification of recombinant proteins

A DNA fragment corresponding to the entire coding region of alr4641 was amplified by performing PCR using the PrxA forward primer 5′-CATATGTCCATCACCTA-3′ (*Nde*I site underlined) and the PrxA reverse primer 5′-CTCGAGTTACACAGCAGCGAAGTA-3′ (*Sac*I site underlined). The PCR product was cloned into the pET28 vector (Novagen, Darnstadt, Germany). A clone confirmed by DNA sequencing was transformed into the BL21DE3 *Escherichia coli* strain (Novagen). The recombinant clones obtained were grown in kanamycin-supplemented medium to an optical density (OD) of 0.3–0.4 and protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h. The recombinant proteins were purified using Hitrap columns as recommended by Healthcare. Imidazol was removed from purified proteins using PD10 columns (Healthcare, Orsay, France). Proteins were concentrated on Vivaspin columns and used for subsequent analyses.

For SrxA production purposes, a DNA fragment corresponding to the entire coding region of asl4146 was amplified by performing PCR using the SrxA forward primer 5′-GAATTCATGGTTAGGGTACAAGAAATT-3′ (*Eco*RI site underlined) and the SrxA reverse primer 5′-AAGCTTCTACGCTAAGTGCATCTTC-3′ (*Hin*dIII site underlined). The PCR product was cloned into the pMAL-c2X plasmid (BioLabs, Charbonières-les-Bains, France). JM109 *E. coli* strain (Promega) was transformed with the pMAL-c2X-*srxA* plasmid. The recombinant clones obtained were grown in ampicillin-supplemented medium up to an OD of 0.3–0.4 and protein expression was induced by adding 1 mM IPTG for 4 h. Cells were collected by centrifugation and lysed by sonication in a buffer containing Tris-HCl (pH 7.4) and protease inhibitor cocktail (Roche, Every, France). The recombinant proteins were first purified using affinity column (amylose resin, NEB) according to the manufacturer's instructions, and then further purified using PD10 columns (Healthcare). The maltose binding protein tag (MBP) was removed in line with the manufacturer's instructions (Healthcare). The cysteine residue at position 57 of SrxA was changed into a serine residue by performing PCR using the megaprimer strategy (Wu *et al.*, 2005). The primers used for this purpose were: SrxA forward, SrxA reverse and SrxA mut primer 5′-ATATCT ATGGCTACCAG-3′.

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

PrxA and SrxA proteins were fractionated by SDS-PAGE (12.5% gel) and stained using the SeeBand procedure (Euromedex, Souffelweyrshim, France). When SDS-PAGE was performed under nonreducing conditions, 2-mercaptoethanol was omitted from the loading buffer. For

immunoblot analysis, the proteins were transferred onto nitrocellulose membranes for revelation with polyclonal antibodies. Immune complexes were detected with anti-rabbit peroxidase-conjugated secondary antibodies (Promega) and enhanced chemiluminescence reagents (Pierce, Illkich, France). Antibodies raised against overoxidized human *Prx*I ($\alpha$-Prx-$SO_3$; Abcam, Paris, France) were diluted to 1 : 5000. Antibodies specific to Arabidopsis 2-Cys Prx were diluted to 1 : 5000. Antibodies raised against the ferredoxin-$NADP^+$ reductase (FNR) from *Anabaena* PCC 7119 were diluted to 1 : 5000 (Razquin *et al.*, 1996). Antibodies specific for His-tag (Invitrogen) were diluted to 1 : 5000.

### Reactive oxygen species detection

The ROS generated by *Anabaena* cells were analysed after performing reactions with 2,7-DCFH-DA (Molecular Probes, Illkich, France) as described elsewhere (Jang *et al.*, 2004). The fluorescence microscope Nikon Eclipse E800 (Champigny sur Marne, France) was used to detect the fluorescence intensity emitted between 515 and 550 nm.

### Pigment determination

Chlorophyll *a* was determined spectroscopically in methanol extracts. For this purpose, 100 µl of *Anabaena* cell cultures were mixed with 1 ml of methanol and vortexed. Cell debris were pelleted by centrifugation, and the absorbance of the supernatant was measured at 665 nm (OD 655). Chlorophyll concentration was calculated in terms of the formula: $\mu g\ ml^{-1} = 13.43 \times OD655 \times$ dilution factor.

Phycocyanin content was calculated from the difference spectrum of cell cultures determined before and after a 8-min heat treatment at 75°C (Collier & Grossman, 1992).

### Preparation of sulfinic PrxA

Recombinant PrxA (5 $\mu g\ \mu l^{-1}$) was incubated in a 50 µl reaction mixture containing 20 mM dithiothreitol (DTT), 3 mM $H_2O_2$, 50 mM Tris-HCl (pH 6.8) and 100 mM KCl. Oxidation was initiated by adding $H_2O_2$ to the reaction mixture and continued for 30 min at 30°C. The addition of 3 mM $H_2O_2$ to the reaction mixture was repeated three times.

### SrxA activity assays

Sulfinic PrxA (15 $ng\ \mu l^{-1}$) was reduced by adding SrxA (15 $ng\ \mu l^{-1}$) to a 200-µl reaction mixture containing 50 mM Tris-HCl (pH 6.8), and 100 mM KCl, 10 mM DTT, 0.5 mM MgCl2; 1 mM ATP was added to the reaction mixture when required. After a 30-min period of incubation at 30°C, the proteins were precipitated with 10% trichloroacetic acid. Proteins were then subjected to immunoblot analysis with specific antibodies to overoxidized human PrxI ($\alpha$-Prx-$SO_3$; Ambion), or with antibodies to *Arabidopsis* 2-Cys Prx. Before *in vivo* reduction, mid-log *Anabaena* strains were treated with methyl viologen 50 µM for 60 min. The cell cultures were then washed three times in BG11 medium and reincubated for 1 h in BG11 medium to enable them to recover from the stress. The redox state of PrxA was analysed as explained earlier.

### Dataset construction

Prokaryotic homologues of *Anabaena* sp. PCC 7120 SrxA and PrxA were retrieved in February 2010 using the BLASTP program (Altschul *et al.*, 1997) (default parameters) from a local database composed of the 1083 complete prokaryotic (1002 bacterial and 81 archaeal) genomes available at the NCBI (ftp.ncbi.nih.gov). BLAST outputs were checked manually to identify homologues (no arbitrary cut-off *e*-value was used). To obtain a broad sampling of eukaryotic sequences, homologues of Srx and Prx were retrieved using the BLASTP and TBLASTN programs from the *nr* database at the NCBI, from the JGI (http://genome.jgi-psf.org/) and TBestDB databases (http://www.bch.umontreal.ca/pepdb/pep.html). In addition, homologues of three representatives of Rhodophyta were retrieved from the *Porphyra yezoensis* expressed sequences tag (EST) dataset (http://est.kazusa.or.jp/en/plant/porphyra/EST/blast.html), the *Galdieria sulphuraria* genome project (http://genomics.msu.edu/cgi-bin/galdieria/blast.cgi) and the *Cyanidioschyzon merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/blast/blast.html). The Srx and Prx homologues were finally assembled in two datasets. The corresponding sequences were aligned using CLUSTALW 2.0.12 (Larkin *et al.*, 2007). The resulting alignments were then inspected visually and refined manually using the ED program from the MUST package (Philippe, 1993). Before phylogenetic analyses, regions of doubtful homology were removed manually from the alignments using NET from the MUST package (datasets are available on request from L. E. or C. B-A.).

The cellular location of each eukaryotic Srx and 2-Cys Prx from photosynthetic organisms was investigated using information available in the Genpept entries combined with bioinformatic predictions of chloroplast transit peptides (CTP) obtained using various software programs, such as CHLOROP (Emanuelsson *et al.*, 1999), TARGETP (Emanuelsson *et al.*, 2007), IPSORT (Bannai *et al.*, 2002), WOLF PSORT (Horton *et al.*, 2007) and PCLR, Chloroplast Localization Prediction release 0.9 (Schein *et al.*, 2001).

### Phylogenetic analyses

Maximum likelihood (ML) phylogenetic trees were computed with PHYML 3.0 (Guindon & Gascuel, 2003) using the Le and Gascuel (LG) model (Le & Gascuel, 2008) and a gamma correction to take into account the heterogeneity

of the evolutionary rates across sites (using four discrete classes of sites and an estimated alpha parameter). Branch robustness of the resulting trees was estimated using the nonparametric bootstrap procedure implemented in PHYML using 100 replicates of the original dataset and the same parameters as for tree reconstruction. Additional ML phylogenetic analyses were performed using TREEFINDER and the same evolutionary model and parameters as for PHYML (Jobb *et al.*, 2004). Bayesian trees were computed using MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003) with a mixed amino acid substitution model and a gamma correction (using four discrete classes of sites and an estimated alpha parameter). The Markov chain Monte Carlo search was run with four chains on 1 000 000 generations, and the trees were sampled every 100 generations (the first 2500 trees were discarded as 'burning' trees).

## Results

### *srxA* and *prxA* genes are induced in response to oxidative stress

The ORF Asl4146 (SrxA) present in *Anabaena* genome (http://www.kazusa.or.jp/cyano/) encodes a protein that is homologous to eukaryotic Srxs (Kaneko *et al.*, 2001). SrxA is an 87-residue protein with predicted molecular mass of 9.7 kDa and a predicted isoelectric point of 9.39. It shows higher levels of identity with proteins found in the land plant *Arabidopsis thaliana* (59%) than with yeast (42%) and human homologues (40%). The transcription of *srxA* gene is induced when *Anabaena* cells are challenged by oxidative conditions such as methyl viologen treatment (Fig. 1a) and strong light stress (Fig. 1b). The expression of the *prxA* gene encoding the *Anabaena* 2-Cys Prx was also induced under the same conditions (Fig. 1). It is worth noting here that the results obtained in Fig. 1 could also indicate that a possible degradation of *srxA* and *prxA* mRNAs is inhibited upon oxidative stress.

A mutant (*ΔsrxA*) from which the *srxA* gene was deleted was constructed, as explained in the Materials and Methods section. A PCR analysis indicated that the mutant was com pletely segregated (data not shown). Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) analyses performed with RNAs extracted from this mutant showed that, contrary to what was observed for the wild type, the *srxA* transcripts were not detected at all when mutant cells were grown under oxidant conditions (Fig. 2a). This finding further confirmed that the *srxA* gene was completely inactivated in the mutant. When cells were exposed to high light intensities or hydrogen peroxide stress, the growth rate of the *ΔsrxA* mutant did not differ significantly from that of the wild-type strain. However, when the strains were concomitantly subjected to strong light and hydrogen peroxide stresses, the mutant showed a much slower rate of growth

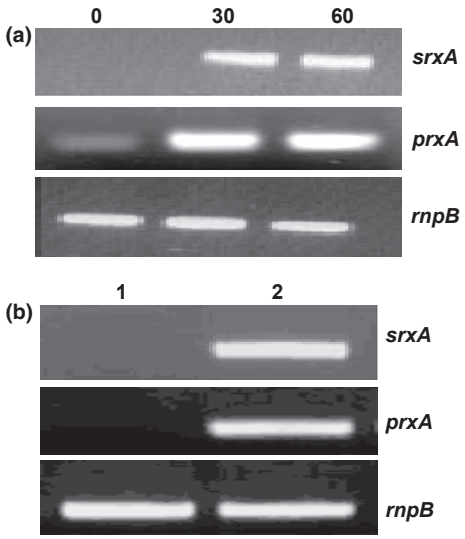


**Fig. 1** *srxA*, encoding a prokaryotic sulfiredoxin, is induced under oxidant conditions. (a,b) Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *srxA* and *prxA* gene transcription. RNA was collected from cells grown in BG11 medium (a, column 0) or in BG11 incubated for 30 min (a, column 30), or 1 h (a, column 60) with 50 μM of methyl viologen (a), or 1 h after transferring the cell culture from normal lighting conditions (b, column 1) to high light (b, column 2). Samples were collected during the exponential phase of the PCR. All RT-PCR experiments were performed in triplicate, and similar results were consistently obtained. Expression of the *rnpB* gene was used as the control assay.

than the wild type strain (Fig. 2b). Deletion of the *srxA* gene led to the strong accumulation of ROS (Fig. 3), which indicates that the *ΔsrxA*mutant is less able than the wild type to reduce ROS. To further characterize the phenotype of the *ΔsrxA* mutant, the light-harvesting pigments chlorophyll *a* and phycocyanin were quantified, because the mutant was found to have a less green appearance than the wild-type strain. Photobleaching has been reported to occur in cyanobacteria in response to high light stress (Andreeva *et al.*, 2007; Jeanjean *et al.*, 2008). When exposed to strong illumination conditions, the mutant cells were found to contain 75% less Chl*a* and 60% less phycocyanin than the wild-type cells (Table 1).

### SrxA reduces sulfinic PrxA *in vitro*

To investigate the activity of SrxA, the *srxA* coding region was overexpressed in *E. coli* as a translation fusion protein with the MBP. After an amylose-affinity chromatography purification step, the recombinant protein recovered in the soluble fraction was found to be relatively homogeneous (Fig. 4a). His-tagged PrxA was expressed in *E. coli* and purified by performing Nickel-affinity chromatography. Under reducing conditions, the monomeric form of the recombinant protein, which was the predominant form observed, had the expected size of 25.5 kDa (which corresponds to PrxA at 25 kDa and the His-tag at 0.5 kDa) (Fig. 4b).

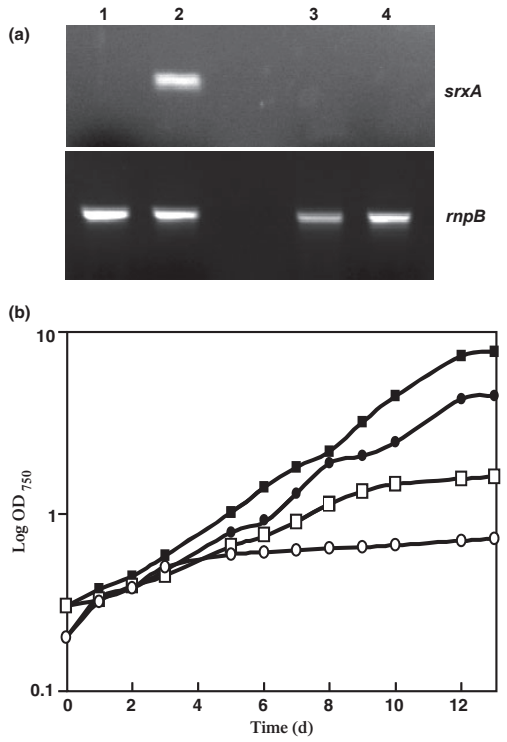


**Fig. 2** Characterization of the *ΔsrxA* mutant. (a) Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *srxA* gene transcription in the *ΔsrxA* mutant. RNA from the wild-type strain (columns 1 and 2) or from the *ΔsrxA* mutant (columns 3 and 4) was collected from cells grown in BG11 medium (columns 1 and 3) or in BG11 incubated for 1 h with 50 μM of methyl viologen (columns 2 and 4). Samples were collected during the exponential phase of the PCR. All RT-PCR experiments were performed in triplicate, and similar results were consistently obtained. Expression of the *rnpB* gene was used as the control assay. (b) Representative experiment showing growth of the wild-type (squares) and *ΔsrxA* (circles) mutant strains under normal lighting in BG11 without hydrogen peroxide ($H_2O_2$, closed symbols), or at high light in BG11 medium with 50 μM of $H_2O_2$ (open symbols).

To examine the ability of SrxA to catalyse the reduction of the sulfinylated form of 2 Cys-Prx, PrxA was subjected to overoxidation, as described in the Materials and Methods section. Overoxidized PrxA is denoted Ox-PrxA. The sulfinylation of PrxA was assessed using antibodies ($\alpha$-Prx-$SO_3$) directed against the sulfonic form of the human 2-Cys Prx. These antibodies recognize purified Ox-PrxA (Fig. 4c). These antibodies were therefore used to monitor the possible reduction of Ox-PrxA by SrxA in the presence of ATP and $Mg^{2+}$. Under these conditions, the band revealed by $\alpha$-Prx-$SO_3$ disappeared completely after a 30-min period of incubation at 30°C, which indicates that SrxA is able to reduce the sulfinic form of PrxA. As in the case of the eukaryotic Srxs, the action of SrxA depends on the presence of ATP: only the overoxidized form (Ox-PrxA) was detected when ATP was omitted from the reaction mixture (Fig. 4c). All the eukaryotic Srx enzymes described so far contain a conserved cysteine residue, which is required for Prx-reduction. In *Anabaena* Srx, the conserved cysteine is $Cys^{57}$. Replacing SrxA $Cys^{57}$ by a serine residue totally abolished the reductase activity (Fig. 4d), which provides further evidence that the catalytic mechanism involving SrxA is similar to that involving eukaryotic Srxs.

## SrxA reduces the sulfinic form of PrxA *in vivo*

In order to determine whether SrxA is active *in vivo*, PrxA oxido-reduction was compared between the wild strain and the *ΔsrxA* mutant. In the wild type, PrxA was converted into a sulfinic form (Ox-PrxA) after a 30-min period of incubation with methyl viologen. Ox-PrxA was converted into its reduced form once the stress had been removed (Fig. 5a). However, in the *ΔsrxA* mutant, the SOOH form of PrxA accumulated and was not converted into the reduced form (Fig. 5b), which clearly indicates that SrxA is involved in PrxA-Cys-SOOH recycling processes *in vivo*. The evidence obtained here that *Anabaena* contains an active Srx challenges the belief that these enzymes play an important role in eukaryotes alone, and shows that they are involved in oxidative stress responses in cyanobacteria.

## 2-Cys Prxs are more widely distributed than sulfiredoxins

To investigate the evolution of 2-Cys Prxs and Srxs, an extensive phylogenetic analysis of these proteins was performed. A survey of sequences databases yielded 1329 Prxs harboring the two cysteine residues characteristic of 2-Cys Prxs (at positions 56 and 178 in the *Anabaena* PrxA sequence). 2-Cys Prxs are present in the complete genomes of many prokaryotes (including all genomes of the Cyanobacteria) and in those of most major lineages of Eucarya for which genomic data are available (Metazoa, Fungi, Amoebozoa, Excavata, Alveolata, Heterokonta and Plantae (i.e. Glaucophytes, Rhodophyta, green algae and land plants; Fig. 6). Phylogenetic analyses showed that the 2-Cys Prx sequences can be divided into two groups: one containing most of the bacterial and archaeal sequences, and the other consisting of sequences from Cyanobacteria, Eucarya and a few other bacteria. Interestingly, only sequences from the latter group harbored the two specific motifs (GG(LIV)G and (YF)(FWL)) previously reported to be a signature of these enzymes' ability to undergo overoxidation (Wood *et al.*, 2003). Accordingly, these sequences will be referred subsequently as sensitive 2-Cys Prxs. These motifs are located at positions 99–102 and 199–200 in the *Anabaena* PrxA sequence, respectively. By contrast, the taxonomic distribution of Srxs is much narrower than that of Prxs. Only 109 Srx homologues belonging to Cyanobacteria and to some eukaryotic lineages (i.e. Metazoa, Fungi, Amoebozoa and land plants) were detected (Fig. 6), in agreement with previous data (Basu & Koonin, 2005).

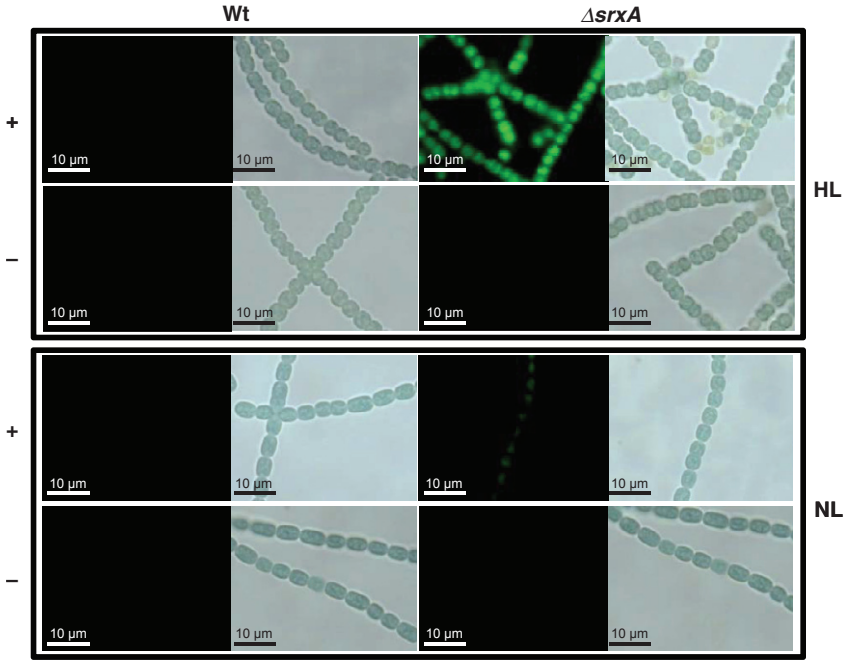


**Fig. 3** SrxA contributes to oxidative stress response. Fluorescence micrographs (right columns), and light transmission micrographs (left columns), of *Anabaena* PCC 7120 wild-type strain (Wt) or *ΔsrxA* mutant *(ΔsrxA)*. Mid-log grown cells were incubated under either under normal (NL) or high light conditions (HL) for 24 h. They were then treated (+) or not (–) with the reactive oxygen species (ROS)-detecting probe CM-$H_2$DCFDA. This experiment was performed with three *ΔsrxA* mutant strains obtained from three independent conjugation events, and similar results were obtained.

**Table 1** Light-dependent decrease in the chlorophyll *a* (Chl*a*) and phycocyanin contents of *Anabaena* wild-type (Wt) and *ΔsrxA* mutant cells

| Cell type and growth conditions | Amount of Chl*a*[1] | Amount of Phycocyanin[2] |
|---|---|---|
| Wt, NL | 5.5 ± 0.32 | 250 ± 12 |
| *ΔsrxA*, NL | 4.9 ± 0.28 | 224 ± 15 |
| Wt, HL | 4.4 ± 0.19 | 183 ± 11 |
| *ΔsrxA*, HL | 1.1 ± 0.25 | 70 ± 9 |

Mid-log grown cells were incubated for a further 48 h under either normal (NL: 40 μmol $m^{-2}$ $s^{-1}$) or higher light intensities (HL: 150 μmol $m^{-2}$ $s^{-1}$).
[1]Expressed as micrograms per OD750 unit of cell suspension.
[2]Expressed as $((\Delta OD_{620} - \Delta OD_{750}) \times 10\,000)/OD_{750}$. $\Delta OD_{620}$ and $\Delta OD_{750}$ are the differences between the OD values recorded at the respective wavelengths before and after incubating the cell suspensions at 75°C (Collier & Grossman, 1992).
Values are means of four independent determinations (± SD).

However, based on the present genomic data, Srxs can now be said to occur in green and red algae, glaucophytes and representatives of the large protist group of Excavates (Fig. 6). However, no Srx homologues were detected in Chromalveolata (i.e. Alveolata, Heterokonta, Haptophyta or Cryptophyta).

In order to obtain further insights into the evolutionary history of these two proteins, an accurate phylogenetic analysis was performed on a subset of Srx and sensitive 2-Cys Prx sequences reflecting the taxonomic diversities of these enzymes. The resulting maximum likelihood and Bayesian trees showed similar topologies; both Srxs and sensitive 2-Cys Prxs trees show two well distinct clusters: the first group contains most of the eukaryotic sequences (including some Plantae sequences and a few bacterial sequences in the case of Prxs, probably resulting from recent horizontal gene transfer (HGT) events), whereas the second gathers all the cyanobacterial sequences and other Plantae sequences (BV = 87% and PP = 1.0 in Srx, and BV = 97% and PP = 1.0 in Prx, Fig. 6). Therefore, Plantae representatives can be said to differ from other eukaryotes in that they are the only lineage which harbors Srxs and sensitive 2-Cys Prxs sequences from both clusters.

The relationships between eukaryotic Srxs and between sensitive 2-Cys Prxs sequences are poorly resolved (BV < 50% and PP < 0.5, Fig. 6). However, the fact that these enzymes are present in most eukaryotic lineages suggests that Srxs and sensitive 2-Cys Prxs have existed for a long time in this domain, and that they were probably present in the last common ancestor of eukaryotes (LECA, closed squares and triangles in Fig. 7c). This indicates that the few plants harboring sequences of this kind (such as the glaucocystophyte *Cyanophora paradoxa* and two green algae, *Chlamydomonas reinhardtii* and *Volvox carteri*, Fig. 6) have inherited them from LECA (closed squares and triangles, Fig. 7). Upon carefully examining the sequences forming the second cluster, it was observed that both 2-Cys Prxs and Srxs are present in most cyanobacterial lineages, especially in deep branching ones such as *Synechococcus* sp. JA-2-3Ba2-13 (Fig. 6). This suggests that sensitive 2-Cys Prxs and Srxs may have existed in cyanobacteria for a long time and that they may have been present in their last common ancestor (open squares and triangles in Fig. 7b). It is worth noting that most Plantae Srxs and sensitive 2-Cys Prxs were found to be closely related to cyanobacterial sequences and

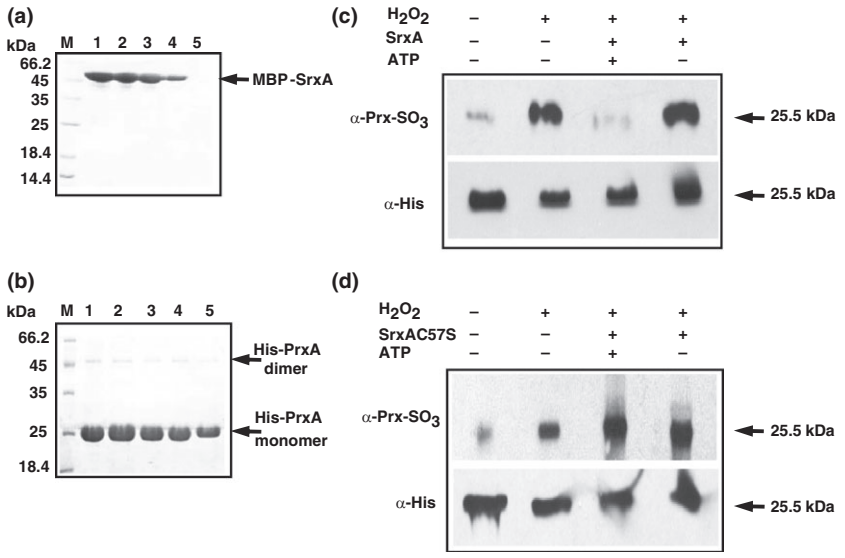


**Fig. 4** SrxA reduces Ox-PrxA *in vitro* (a,b) Purification of recombinant SrxA and PrxA proteins. SrxA was purified as a maltose-binding protein (MBP)-tagged protein and PrxA as a His-tagged fusion protein. After an affinity chromatography purification step, MBP-SrxA (a) and His-PrxA (b) were further purified using PD10 columns. Proteins eluted showed good levels of homogeneity in the 4th (a) and 5th (b) elution fractions. Proteins were separated by performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5% acrylamide gel) and stained with SeeBand. Lanes: M, molecular size markers; 1–5, PD10 elution fractions. (c,d) Western blot analysis of the sulfinic form of PrxA. Overoxidized PrxA was obtained as specified in the Materials and Methods section. *In vitro* reduction assays were performed with SrxA (c) or SrxAC57S (d) on the Ox-PrxA thus obtained. Untreated PrxA protein was used as the control substance ($-H_2O_2$ lane). Where indicated, ATP was omitted from the reaction mixtures. Western blotting performed with His-probe antibodies (Invitrogen) served to check the amounts of protein used in each assay.

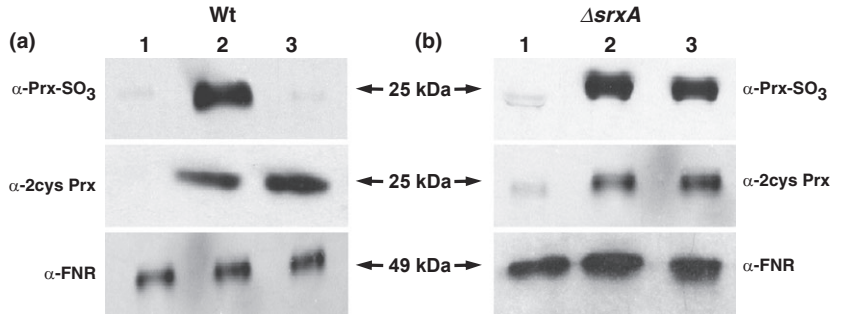


**Fig. 5** SrxA recycles Ox-PrxA *in vivo* Western blot analysis of PrxA in the wild-type *Anabaena* strain (a) and in the *ΔsrxA* mutant (b). Cells were grown in BG11 medium up to the mid-log growth phase (1) and incubated with 50 μM methyl viologen for 1 h (2). Methyl viologen treated cells were left to recover from the stress as explained in the Materials and Methods section (3). Samples of 50 μg of proteins were used in each assay. The antibodies directed against the 2-Cys Prx from *Arabidopsis thaliana* served to detect the PrxA protein, and anti-ferredoxin-$NADP^+$ reductase (FNR) antibodies served to control the amount of protein used in each assay.

to be only distantly related to sequences found in other eukaryotes (Fig. 6). This indicates that these Plantae sequences have a different evolutionary origin. The most parsimonious hypothesis accounting for the close relationship between Plantae and cyanobacterial sequences is that a HGT has occurred between those two lineages.

## Discussion

The present *in vivo* and in *vitro* analysis of *Anabaena* 2-Cys Prx (PrxA) oxidation processes showed the presence in this organism of a sulfiredoxin (SrxA), which is similar to those present in eukaryotes. SrxA reduces Ox-PrxA via a similar mechanism to that occurring in eukaryotes and thus helps *Anabaena* to cope with oxidative stress. It was therefore concluded that Srxs are not restricted to eukaryotes alone, and that SrxA is a prokaryotic member of the Srxs enzyme family. As some other cyanobacterial strains have sulfiredoxin homologs (triangles in Fig. 7), it is tempting to suggest that sulfinylation and reduction of sensitive 2-Cys Prxs may also occur in these organisms. Transcription of the *srxA* gene can be strongly induced under various conditions resulting in oxidative damage. It is therefore rather surprising that the lack of this gene significantly affects the bacterial growth

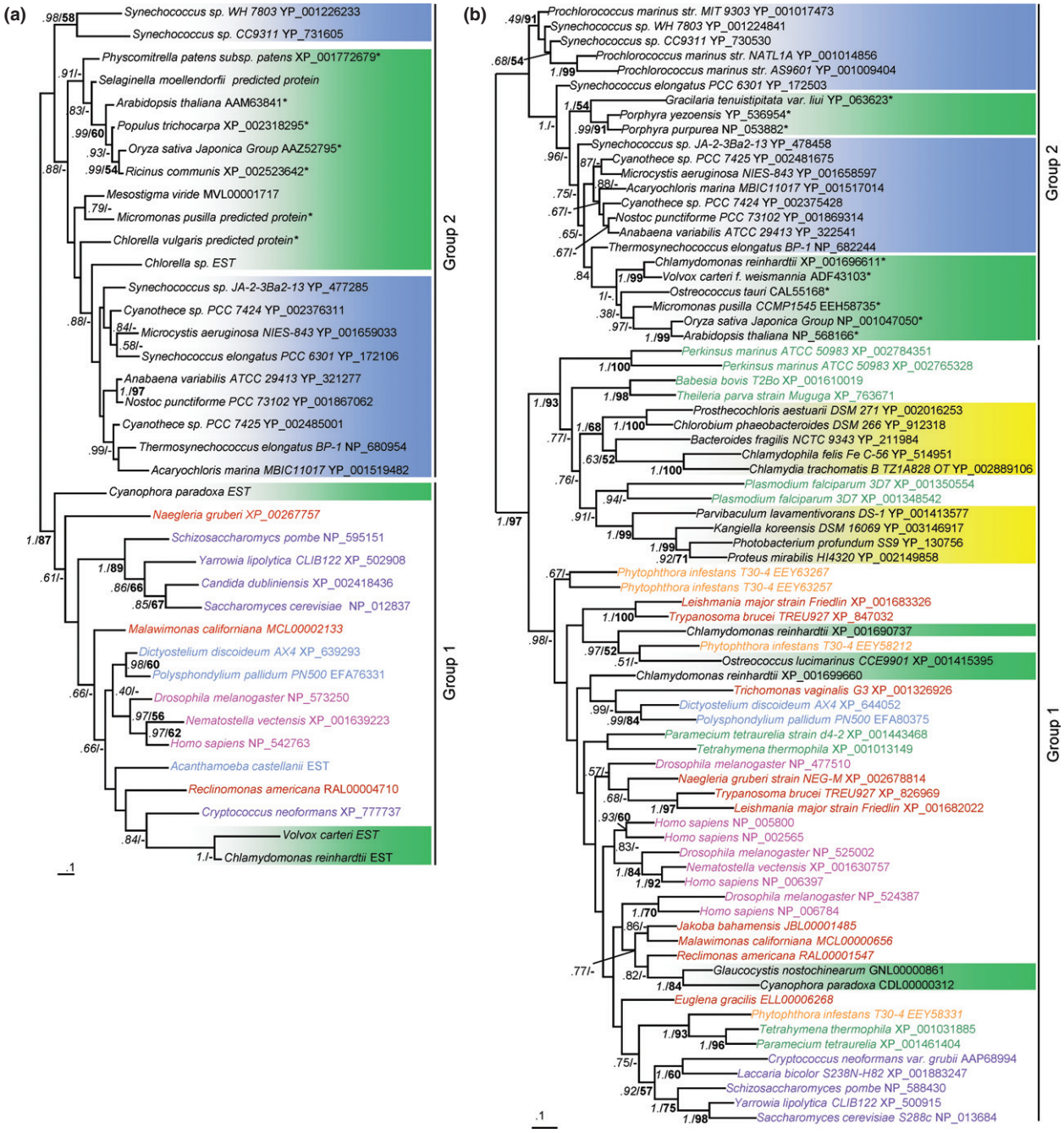


**Fig. 6** SrxA and PrxA are closely related to plant enzymes. Rooted Bayesian trees of Srx (74 positions, 38 sequences) (a) and sensitive 2-Cys Prx (167 positions, 76 sequences) (b) homologues. The Srx tree was rooted using a distant homologue, namely the chromosome partioning ParB protein as an outgroup (Basu & Koonin, 2005). The sensitive 2-Cys Prx phylogeny was rooted using nonsensitive 2-Cys Prx homologues as an outgroup (not shown). Green, blue and yellow boxes give Plantae, cyanobacterial and other bacterial sequences, respectively. Other eukaryotic groups are presented in fuchsia (Metazoa), purple (Fungi), blue (Amoebozoa), dark red (Excavata), dark green (Alveolata) and orange (Heterokonta) fonts. Plant sequences harboring a chloroplastic target peptide are indicated by an asterisk. Numbers at nodes are posterior probabilities (PP)/bootstrap values (BV). For the sake of clarity, only PP and BV values > 0.5% and 50%, respectively are indicated. The scale bars give the average number of substitutions per site.

rates only under accumulated stresses. Similar results have been obtained with the Srx mutant of *A. thaliana,* the growth of which was impaired only under high light and low temperature conditions (Rey *et al.*, 2007). The Srxs are important players in the regulation of Prxs, but their role becomes essential only under severe oxidative conditions.

Our sequence database survey showed that both sensitive 2-Cys Prxs and Srxs have a similar evolutionary history,

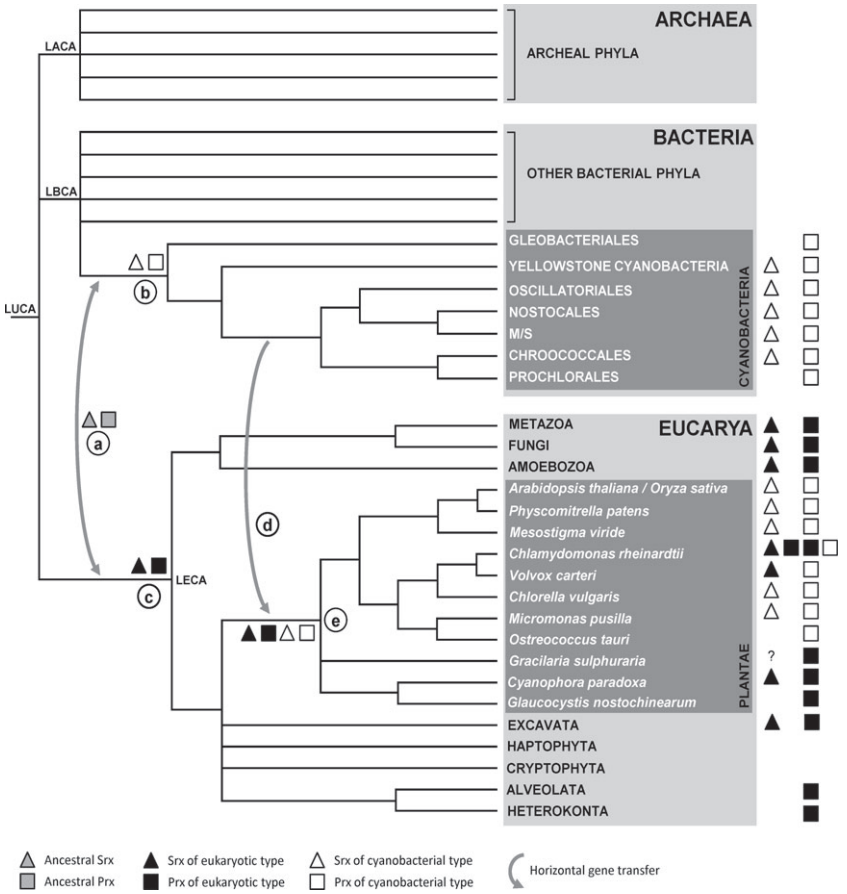


**Fig. 7** Evolution of Srxs and sensitive 2-Cys Prxs. Possible scenario of the origin and evolutionary history of Srx and sensitive 2-Cys Prx sequences. The tree gives the three domains of cell life (Archaea, Eucarya and Bacteria). LECA, LACA, LBCA and LUCA are, respectively: the Last Eukaryotic, Archaeal, Bacterial and Universal Common Ancestor. The Srx (tinted triangle) and sensitive 2-Cys Prx (tinted square) sequences originated from the ancestor of either Cyanobacteria or Eucarya and were horizontally transferred to the other lineage before their diversification (a). Srx and sensitive 2-Cys Prx sequences were conserved during evolution and were present in the ancestor of Cyanobacteria (b, open triangle and square) and in the ancestor of Eucarya (c, closed triangle and square). The cyanobacterial Srx and 2-Cys Prx sequences were acquired secondarily by Plantae via the chloroplastic endosymbiosis (d). After this event, either eukaryotic or cyanobacterial copies of Srx and 2-Cys Prx were lost during the evolution and diversification of the present-day Plantae lineages (e). The late acquisition of sensitive 2 Cys-Prxs is not indicated (see text for details).

which means that they have coevolved. This result is not surprising as these enzymes are functionally linked. It is worth mentioning that all the genomes containing a Srx homologue also encode a sensitive 2-Cys Prx homologue (Fig. 7). However, the opposite is not true because some sensitive 2-Cys Prx coding genes were found to exist in genomes devoid of *srx* homologues (such as *Synechocystis* PCC 6803, and Chromalveolata, Fig. 6 and Table S1). This suggests either that some other hitherto uncharacterized proteins may reduce sulfinic Prxs, or that the presence of the overoxidation motif in a 2-Cys Prx does not necessarily mean that this protein is prone to sulfinylation. The fact that the 2-Cys Prx of *Synechocystis* PCC 6803, which lacks Srx, is much less sensitive to overoxidation than the 2-Cys Prx of *Anabaena*-PrxA (Pascual *et al.*, 2010) argues in favor of the second possibility.

The taxonomic distribution of 2-Cys Prxs and Srxs observed here is rather puzzling, and raises questions about the origin of these enzymes. It has been previously suggested that Cyanobacteria may have acquired their Srxs via an HGT event from plants (Basu & Koonin, 2005). Since the latter study, many more genome sequences have become available, and the evolutionary history of these proteins was therefore reassessed here. Our data indicate that both Srx and sensitive 2-Cys Prx were probably present in the last common ancestor of Eukaryotes (Fig. 7c), as well as in the last common ancestor of present-day Cyanobacteria (Fig. 6b). This strongly suggests that an ancient HGT event may have occurred between these two lineages before their diversification (tinted square and triangle, Fig. 7a). In addition to this event, a second HGT event may have occurred between cyanobacteria and plants, which would explain the presence of a

cyanobacterial copy of these enzymes in plants (Fig. 7d): the presence of two distinct versions of 2-Cys Prxs and Srxs in plants does not support the hypothesis that an HGT may have occurred from plants to cyanobacteria, but rather suggests that one of the two Srx and 2-Cys Prx copies may be of chloroplastic origin. The presence of a chloroplastid target peptide in plant sequences closely related to cyanobacterial Srx and sensitive 2-Cys Prx, and the fact that these enzymes were found to be localized in the chloroplast in several species (Baier & Dietz, 1997; Rey *et al.*, 2007), are consistent with this evolutionary scenario. The diversity of the Srx and 2-Cys Prx repertoire observed in present-day plants therefore results from the subsequent loss of one of the two copies inherited from either Cyanobacteria or LECA (either closed or open squares and triangles, Fig. 7), or both.

Why do some cyanobacteria require a Srx and not others? The answer to this question will shed light on the strategies developed by these organisms to cope with oxidative stress. If having a Srx serves the same purpose in both cyanobacteria and eukaryotes, it might be possible to conclude that in cyanobacteria strains carrying a Srx, the peroxide signaling processes may involve a 2-Cys Prx. Therefore, studying the peroxide perception mechanisms and those involved in the regulation of the appropriate responses between cyanobacteria with and without sulfiredoxin is the next challenge that needs to be met in this field.

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## References

**Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997**. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

**Andreeva A, Abarova S, Stoitchkova K, Picorel R, Velitchkova M. 2007**. Selective photobleaching of chlorophylls and carotenoids in photosystem I particles under high-light treatment. *Photochemistry and Photobiology* **83**: 1301–1307.

**Baier M, Dietz KJ. 1997**. The plant 2-Cys peroxiredoxin BAS1 is a nuclear-encoded chloroplast protein: its expressional regulation, phylogenetic origin, and implications for its specific physiological function in plants. *Plant Journal* **12**: 179–190.

**Bannai H, Tamada Y, Maruyama O, Nakai K, Miyano S. 2002**. Extensive feature detection of *N*-terminal protein sorting signals. *Bioinformatics* **18**: 298–305.

**Basu MK, Koonin EV. 2005**. Evolution of eukaryotic cysteine sulfinic acid reductase, sulfiredoxin (Srx), from bacterial chromosome partitioning protein ParB. *Cell Cycle* **4**: 947–952.

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

**Bozonet SM, Findlay VJ, Day AM, Cameron J, Veal EA, Morgan BA. 2005**. Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide. *Journal of Biological Chemistry* **280**: 23319–23327.

**Cai YP, Wolk CP. 1990**. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *Journal of Bacteriology* **172**: 3138–3145.

**Chang TS, Jeong W, Woo HA, Lee SM, Park S, Rhee SG. 2004**. Characterization of mammalian sulfiredoxin and its reactivation of overoxidized peroxiredoxin through reduction of cysteine sulfinic acid in the active site to cysteine. *Journal of Biological Chemistry* **279**: 50994–51001.

**Collier JL, Grossman AR. 1992**. Chlorosis induced by nutrient deprivation in *Synechococcus* sp. strain PCC 7942: not all bleaching is the same. *Journal of Bacteriology* **174**: 4718–4726.

**Dietz KJ. 2007**. The dual function of plant peroxiredoxins in antioxidant defence and redox signaling. *Sub-cellular Biochemistry* **44**: 267–294.

**Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007**. Locating proteins in the cell using TargetP, SignalP and related tools. *Nature Protocols* **2**: 953–971.

**Emanuelsson O, Nielsen H, von Heijne G. 1999**. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Science* **8**: 978–984.

**Findlay VJ, Tapiero H, Townsend DM. 2005**. Sulfiredoxin: a potential therapeutic agent? *Biomedicine and Pharmacotherapy* **59**: 374–379.

**Guimaraes BG, Souchon H, Honore N, Saint-Joanis B, Brosch R, Shepard W, Cole ST, Alzari PM. 2005**. Structure and mechanism of the alkyl hydroperoxidase AhpC, a key element of the *Mycobacterium tuberculosis* defense system against oxidative stress. *Journal of Biological Chemistry* **280**: 25735–25742.

**Guindon S, Gascuel O. 2003**. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696–704.

**Hall A, Karplus PA, Poole LB. 2009**. Typical 2-Cys peroxiredoxins – structures, mechanisms and functions. *FEBS Journal* **276**: 2469–2477.

**Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K. 2007**. WoLF PSORT: protein localization predictor. *Nucleic Acids Research* **35**(Web Server issue): W585–W587.

**Jacob C, Holme AL, Fry FH. 2004**. The sulfinic acid switch in proteins. *Organic & Biomolecular Chemistry* **2**: 1953–1956.

**Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW *et al.* 2004**. Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* **117**: 625–635.

**Jeanjean R, Latifi A, Matthijs HC, Havaux M. 2008**. The PsaE subunit of photosystem I prevents light-induced formation of reduced oxygen species in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochimica et Biophysica Acta* **1777**: 308–316.

**Jeong W, Park SJ, Chang TS, Lee DY, Rhee SG. 2006**. Molecular mechanism of the reduction of cysteine sulfinic acid of peroxiredoxin to cysteine by mammalian sulfiredoxin. *Journal of Biological Chemistry* **281**: 14400–14407.

**Jobb G, von Haeseler A, Strimmer K. 2004**. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evolution Biology* **4**: 18.

**Jonsson TJ, Lowther WT. 2007**. The peroxiredoxin repair proteins. *Subcellular Biochemistry* **44**: 115–141.

**Jonsson TJ, Murray MS, Johnson LC, Lowther WT. 2008a**. Reduction of cysteine sulfinic acid in peroxiredoxin by sulfiredoxin proceeds directly through a sulfinic phosphoryl ester intermediate. *Journal of Biological Chemistry* **283**: 23846–23851.

**Jonsson TJ, Tsang AW, Lowther WT, Furdui CM. 2008b**. Identification of intact protein thiosulfinate intermediate in the reduction of cysteine sulfinic acid in peroxiredoxin by human sulfiredoxin. *Journal of Biological Chemistry* **283**: 22890–22894.

**Kaneko T, Nakamura Y, Wolk CP, Kuritz T, Sasamoto S, Watanabe A, Iriguchi M, Ishikawa A, Kawashima K, Kimura T *et al.* 2001**. Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. *DNA Research* **8**: 205–213; 227–253.

**Konig J, Lotte K, Plessow R, Brockhinke A, Baier M, Dietz KJ. 2003**. Reaction mechanism of plant 2-Cys peroxiredoxin. Role of the *C* terminus and the quaternary structure. *Journal of Biological Chemistry* **278**: 24409–24420.

**Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R *et al.* 2007**. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.

**Le SQ, Gascuel O. 2008**. An improved general amino acid replacement matrix. *Molecular Biology and Evolution* **25**: 1307–1320.

**Liu XP, Liu XY, Zhang J, Xia ZL, Liu X, Qin HJ, Wang DW. 2006**. Molecular and functional characterization of sulfiredoxin homologs from higher plants. *Cell Research* **16**: 287–296.

**Pascual MB, Mata-Cabana A, Florencio FJ, Lindahl M, Cejudo FJ. 2010**. Overoxidation of 2-Cys peroxiredoxin in prokaryotes: cyanobacterial 2-Cys peroxiredoxins sensitive to oxidative stress. *Journal of Biological Chemistry* **285**: 34485–34492.

**Philippe H. 1993**. MUST, a computer package of Management Utilities for Sequences and Trees. *Nucleic Acids Research* **21**: 5264–5272.

**Poole LB. 2007**. The catalytic mechanism of peroxiredoxins. *Sub-cellular Biochemistry* **44**: 61–81.

**Razquin P, Fillat MF, Schmitz S, Stricker O, Böhme H, Gomez-Moreno C, Peleato ML. 1996**. Expression of ferredoxin-NADP$^+$ reductase in heterocysts from *Anabaena* sp. *Biochemical Journal* **15**: 157–160.

**Rey P, Becuwe N, Barrault MB, Rumeau D, Havaux M, Biteau B, Toledano MB. 2007**. The *Arabidopsis thaliana* sulfiredoxin is a plastidic cysteine-sulfinic acid reductase involved in the photooxidative stress response. *Plant Journal* **49**: 505–514.

**Ronquist F, Huelsenbeck JP. 2003**. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

**Schein AI, Kissinger JC, Ungar LH. 2001**. Chloroplast transit peptide prediction: a peek inside the black box. *Nucleic Acids Research* **29**: E82.

**Wood ZA, Poole LB, Karplus PA. 2003**. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* **300**: 650–653.

**Wu W, Jia Z, Liu P, Xie Z, Wei Q. 2005**. A novel PCR strategy for high-efficiency, automated site-directed mutagenesis. *Nucleic Acids Research* **33**: e110.

## Supporting Information

Additional supporting information may be found in the online version of this article.

**Table S1** The taxonomic distribution of sulfiredoxin (Srx) and sensitive 2-Cys peroxiredoxins (Prx) homologues in all cyanobacterial complete genomes available in February 2010 and in a subset of complete genomes representative of main eukaryotic lineages

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