

Molecular and functional characterization of sulfiredoxin homologs from higher plants

Xian Peng Liu^{1,2}, Xue Ying Liu^{1,2}, Juan Zhang^{1,2}, Zong Liang Xia^{1,2}, Xin Liu¹, Huan Ju Qin¹, Dao Wen Wang¹

¹The State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101, China; ²Graduate University of Chinese Academy of Sciences, Yuquan Road, Shijingshan District, Beijing 100039, China

By reducing cysteine-sulfinic acid in oxidized peroxiredoxin, sulfiredoxin (Srx) plays an important role in oxidation stress resistance in yeast and human cells. Here, we report the first molecular and functional characterization of Srx homolog from higher plants. Bioinformatic analysis revealed the presence of potential Srx encoding sequences in both monocot and dicot plant species. Putative plant Srx proteins exhibited significant identities to their orthologs from yeast and human, and contained the conserved signature sequence and residues essential for catalysis. However, unlike yeast and human orthologs, plant Srxs were all predicted to possess chloroplast transit peptide in their primary structure. The Srx proteins from *Arabidopsis* and rice (designated as AtSrx and OsSrx, respectively) complemented functional deficiency of Srx in the *SRX1* deletion yeast cells. A GFP fusion protein of AtSrx was targeted to chloroplast in *Arabidopsis* mesophyll protoplast. AtSrx transcription occurred in both vegetative and reproductive organs, and the highest transcript level was detected in leaves. Under oxidation stress, AtSrx transcript level was substantially increased, which paralleled with enhanced transcription of 2-Cys peroxiredoxins that have been found essential in maintaining chloroplast redox balance. In addition to oxidation stress, osmotic/water deficit or cold treatments also raised AtSrx transcript level. Consistent with above findings, the knock-out mutant of *AtSrx* was significantly more susceptible to oxidation stress than wild type *Arabidopsis* plant. Taken together, the results of this work indicate the existence of functional Srx homolog in higher plants that is essential for plants to cope with oxidation stress.

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Introduction

The sulfur atom in cysteine (Cys) is sensitive to oxidation by a variety of oxidants including reactive oxygen species (ROS) that come from environment or are produced as byproduct of cellular metabolism [1, 2]. Consequently, the sulfhydryl group (-SH) of cysteine residue in cellular proteins can exist in several different oxidation states [3]. Reversible oxidation of cysteine residues to form disulfide bridge (-S-S-), which is an important posttranslational modification and contributes to regulation of protein func-

tion, has been studied extensively [4]. In contrast, the oxidation of Cys-SH to Cys-SOH (sulfenic acid), Cys-SO₂H (sulfinic acid) or Cys-SO₃H (sulfonic acid) has not been well investigated [3]. While it is known that cysteine disulfide and Cys-SOH are reduced by glutathione and thioredoxin [4], the proteins involved in reducing Cys-SO₂H have only begun to be analyzed very recently. In 2003, the yeast protein sulfiredoxin (Srx) was identified with the capacity to reduce Cys-SO₂H in overoxidized peroxiredoxin (Prx) Tsa1 in the presence of ATP and Mg²⁺ [5]. Subsequently, the mammalian orthologs of Srx were also found to participate in the reduction of Cys-SO₂H, which again involved ATP hydrolysis [6]. Surprisingly, an extensive biochemical investigation has shown that the reduction of Cys-SO₂H by purified human Srx (hSrx) is specific to 2-Cys Prx isoforms [7], which contain two conserved cysteine residues separated by 121 amino acid residues [8]. The high specificity of

Correspondence: Dao Wen Wang

Tel: +86-10-64889380; Fax: +86-10-64854467;

E-mail: dwwang@genetics.ac.cn

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hSrx catalysis is tightly correlated with the specific binding of hSrx to the 2-Cys Prxs [7].

Amino acid sequence comparison has indicated that Srx proteins from eukaryotic organisms share a similar primary structure and contain the conserved signature sequence Phe-Gly/Ser-Gly-Cys-His-Arg [9]. Crystal structure analysis has shown that the overall topology of hSrx is composed of three α helices and one five-stranded, mixed β sheet [9]. While helices $\alpha 1$ and $\alpha 3$ are located at the two ends of the structure, helix $\alpha 2$ is positioned internally and close to the β sheet. The signature sequence, Phe96-Gly97-Gly98-Cys99-His100-Arg101, is contained in the N-terminus of helix $\alpha 2$ and the coil structure immediately before this helix. Within this element, the Gly98-Cys99-His100-Arg101 element is directly involved in ATP binding, and Cys99 is the essential active site residue. In addition to the residues located in

the signature sequence, Arg51 and Lys61 also contributes to ATP binding, and the two residues have been found conserved in yeast and mammalian Srx proteins described thus far [5, 6, 9]. During catalysis, the 2-Cys Prx containing Cys-SO₂H binds specifically to hSrx, the sulfinic group is then activated by reacting with ATP, leading to the formation of a sulfinic phosphoryl ester [9]. The conversion of sulfinic phosphoryl ester to the reduced sulfhydryl group involves additional enzymes and is thiol-dependent [9].

Compared to yeast and mammals, systematic efforts in studying the molecular and biochemical properties of Srx proteins from higher plants have not been reported. In plant cells, ROS are produced during normal metabolic and developmental processes or in response to biotic and abiotic stresses [10]. Chloroplast, which is the site of photosynthesis and home of several important biosynthetic

Table 1 A list of PCR primers used in this work

Primer	Primer sequence ^a	Usage
AtSrx-F (pYES2)	5'-ATC <u>GGATCC</u> ATG GCG AAT TTG ATG ATG-3'	Yeast functional complementation
AtSrx-R (pYES2)	5'-ACT <u>GAA TTC</u> TCA GCG AAG ATG ATG CCT TA-3'	
OsSrx-F (pYES2)	5'-ATT <u>GGA TCC</u> ATG GCG GCG AGC TCG AGC-3'	
OsSrx-R (pYES2)	5'-ATT <u>GAA TTC</u> TCA TCG CAT ATG GTG CCT TA-3'	
p35S-mAtSrx-GFP-F	5'-ACT <u>GTC GAC</u> ATG GGA TCT AGC GGC GGT GTA-3'	Chloroplast targeting
p35S-AtSrx-GFP-F	5'-ACT <u>GTC GAC</u> ATG GCG AAT TTG ATG ATG -3'	
p35S-AtSrx-GFP-R	5'- ATT <u>GGA TCC</u> GCG AAG ATG ATG CCT TAA TG-3'	
AtSrx-F (163)	5'-ATC <u>GGATCC</u> ATG GCG AAT TTG ATG ATG-3'	Construction of pBin35S-AtSrx
AtSrx-R (163)	5'-ACT <u>GAA TTC</u> TCA GCG AAG ATG ATG CCT TA-3'	
LBa1	5'-TGG TTC ACG TAG TGG GCC ATC G-3'	Identification of T-DNA insertion mutant
Salk_015324-LP	5'-AGA CCG TTG ATG CGA ACC AGA-3'	
Salk_015324-RP	5'-AAC CTC CCT TAG GCA TGG ACG G-3'	
EGFP-163-F	5'-ATA <u>CCA TGG</u> ATG GTG AGC AAG GGC GA-3'	Preparation of 35S-GFP cassette
EGFP-163-R	5'-ATT <u>CCC GGG</u> TTA CTT GTA CAG CTC GTC-3'	
TuF	5'-AGA ACA CTG TTG TAA GGC TAA AC-3'	Semiquantitative RT-PCR
TuR	5'-GAG CTT TAC TGT CTC GAA CAT GG-3'	
AtSrx-F	5'-ATG GCG AAT TTG ATG ATG AGA TT-3'	
AtSrx-R	5'-TCA GCG AAT ATG ATG CCT TAA T -3'	
2-Cys PrxA-F	5'-ATG GCG TCT GTT GCT TCT TCA A-3'	
2-Cys PrxA-R	5'-CTA AAT AGC TGA GAA GTA CTC TTT GCT G-3'	
2-Cys PrxB-F	5'-ATG TCA ATG GCG TCT ATA GCT TCT T-3'	
2-Cys PrxB-R	5'-CTA GAT AGC TGA AAA GTA TTC TTT GCT GA -3'	

^a The underlined nucleotides (GGATCC, GAATTC, CCATGG, GTCGAC, CCCGGG) form *Bam*HI, *Eco*RI, *Nco*I, *Sal*I and *Sma*I restriction sites, respectively.

pathways involved in the synthesis of amino acids, fatty acids or nucleotides, is one of the main venues for ROS production in green plant tissues [10]. 2-Cys Prx isoforms have been found to play an essential role in the detoxification of peroxides within chloroplast [11]. Considering the functional relationship between 2-Cys Prx and Srx that has been demonstrated in yeast and animal cells [5, 6, 12], it is important to examine if there is functional Srx homolog in higher plants and whether plant Srx is targeted to chloroplast. In this work, we provide molecular genetic evidence for the presence of functional Srx homolog in both dicot and monocot plants, and discuss the potential role of plant Srx protein under normal or stress conditions.

Materials and Methods

Plant and yeast materials, PCR primers and general molecular methods

Arabidopsis thaliana (ecotype Col-0) and rice (*Oryza sativa* L. cv. 'Nipponbare') plants were maintained in greenhouse. The temperatures for *Arabidopsis* and rice growth were 22 °C and 28 °C, respectively. The standard photoperiod and medium for growing *Arabidopsis* aseptically are 16 h light/ 8 h dark and MS plate supplemented with 2% sucrose, respectively. *Saccharomyces cerevisiae* *SRX1* deletion strain (*MAT alpha*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YKL086w::kanMX4*) was obtained from EUROSCARF (<http://www.uni-frankfurt.de/fb15/mikro/euroscarf/>). Chemicals for yeast complementation experiment (hydrogen peroxide) and osmotic/water deficit stress treatment (polyethylene glycol 8000) were purchased from Sigma. The media for growing *Arabidopsis* seedlings and plants were prepared as previously described [13]. Yeast growth media were prepared according to methods detailed in the website of Invitrogen (http://www.invitrogen.com/content/sfs/manuals/pyes2_man.pdf). The oligonucleotide primers used in this work are listed in Table 1. General molecular methods for manipulating DNA or RNA are those detailed previously [14]. The correctness of DNA cloning experiments was always confirmed by restriction enzyme digestion analysis and DNA sequencing.

Bioinformatic analysis

Previous study has identified a potential homolog of yeast Srx in *Arabidopsis* [5]. This putative protein (GenBank accession No. AAO42977) is encoded by the locus *At1g31170*. Using the deduced coding and protein sequences of *Arabidopsis* Srx homolog as queries, a variety of public nucleic acid and protein databases was searched, which resulted in the finding of potential homologs of yeast Srx in both higher plants and algae species. The identities of putative plant Srxs to their yeast and human orthologs were computed using the MegAlign program of DNASTar (Laser gene). For analyzing conserved elements in primary structure, the deduced amino acid sequences of putative plant Srxs were aligned with those of human and yeast Srx proteins using ClustalW (www.ebi.ac.uk/clustalw/index.html). For predicting chloroplast transit peptide in putative plant Srxs, the ChloroP1.1 and TargetP1.1 programs (<http://www.cbs.dtu.dk/services/>) were used with default options.

Cloning of putative Srx cDNAs from Arabidopsis and rice

Gene specific primers were designed for amplifying cDNAs corresponding to the coding regions of Srxs from *Arabidopsis* and rice. Total RNA samples were isolated from *Arabidopsis* and rice leaves using RNeasy plant mini kit (Qiagen). After further treatment with RNase-free-DNase set (Qiagen) (to remove genomic DNA contamination), the RNA samples were converted into cDNA using M-MLV reverse transcriptase (Promega). PCR experiments using cDNA samples were conducted as detailed previously [15]. The cDNA fragment of expected size was cloned using the pGEM[®]-T Easy plasmid vector (Promega). Positive clones (identified by restriction enzyme digestion analysis and DNA sequencing) were sequenced from both strands. The cDNA sequence of *Arabidopsis* Srx obtained in this work was identical to a previously submitted mRNA sequence (GenBank accession No. BT004731) that is specified by *Arabidopsis* locus *At1g31170*. The cDNA sequence of rice Srx determined in this work was deposited in GenBank with the accession number DQ132851.

Complementation of Srx deficiency in SRX1 deletion yeast cells

To test if putative plant Srx would functionally complement Srx deficiency in yeast cells, the cDNAs of *Arabidopsis* and rice Srxs were ectopically expressed in the *SRX1* deletion yeast strain. The two plant cDNAs were individually cloned into the galactose inducible yeast expression vector pYES2 (Invitrogen), resulting in the constructs pAtSrx and pOsSrx, respectively. The two expression constructs and the control vector pYES2 were each transformed into the *SRX1* deletion cells using a simplified yeast transformation method [16]. The resulted recombinant strains were selected using uracil deficient medium and were further verified by PCR amplification of AtSrx or OsSrx coding regions [17]. The positive colonies and the colony containing pYES2 were streaked onto uracil-free plates that contained 2 % galactose and 2 mM hydrogen peroxide. The plates were then incubated at 28 °C for 72 h before yeast growth was examined and photographed.

Intracellular targeting of putative Arabidopsis Srx

Three DNA constructs (p35S-GFP, p35S-AtSrx-GFP, p35S-mAtSrx-GFP) were prepared for investigating intracellular targeting of *Arabidopsis* Srx using transient expression in *Arabidopsis* mesophyll protoplasts. p35S-GFP was created by cloning the coding region of EGFP (Clontech) down-stream of the 35S promoter in the plasmid vector pJIT163 (<http://www.pgreen.ac.uk/>). p35S-AtSrx-GFP was constructed by cloning the complete coding region of *Arabidopsis* Srx up-stream (and in frame with) the GFP coding region of p35S-GFP. p35S-mAtSrx-GFP was developed similarly as p35S-AtSrx-GFP, except that the coding region of *Arabidopsis* Srx in this construct was truncated by lacking the coding sequence for the first 30 amino acid residues. These residues had been predicted to form a potential chloroplast transit peptide by ChloroP1.1 program. *Arabidopsis* mesophyll protoplasts were isolated [18], transfected with each of above three constructs [19], and examined by dual channel confocal microscopy [20]. The bright field image, GFP fluorescence, and the red autofluorescence of chloroplast from expressing protoplast were recorded simultaneously and compared. Potential colocalization of GFP fluorescence with chloroplast autofluorescence was further analyzed by checking the presence of yellow signal in superimposed images.

Examination of AtSrx transcript levels in Arabidopsis organs and during stress

To compare the relative transcript levels of AtSrx in *Arabidopsis* vegetative and reproductive organs, total RNA samples were isolated from roots, stems, leaves, flowers and siliques, respectively, and were reversely transcribed into cDNAs as described above. The cDNA contents of different reverse transcription mixtures were carefully normalized by amplifying tubulin transcripts [21]. The relative transcript levels of AtSrx in the different organs were then evaluated using the normalized cDNA samples. To monitor the kinetics of above semiquantitative RT-PCR, tubulin transcripts were amplified (using the same set of normalized cDNA samples) with different numbers of amplification cycles (i. e., 24, 28, 32, or 35).

For stress treatment, *Arabidopsis* seedlings (2 weeks old) were transferred into liquid MS medium (supplemented with 1% sucrose). After two day adaptation, oxidation stress was initiated by adding hydrogen peroxide (4 mM final concentration) to the liquid medium [22, 23]. The duration of the treatment was 6 h. Osmotic/water deficit stress was augmented by growing the adapted seedlings in liquid MS medium containing 10% PEG8000 for 8 h [24]. Cold stress was accomplished by growing the adapted seedlings (in liquid MS medium) at 4 °C for 8 h [25]. Following each treatment, the seedlings were immediately harvested and stored in liquid nitrogen. AtSrx transcript levels in the stressed seedlings were evaluated (using semiquantitative RT-PCR) as described above. As positive control for oxidant induced gene expression changes, alterations in the transcript levels of 2-Cys

Tomato	MAN-FVLQLPNNLRLRS-----FTVSSSSSNG-ATPGVPQSA---G
Potato	MAN-FVLQLPNNLRLRS-----FTVSSSSSNG-ATPGVPQSA---G
<i>Arabidopsis</i>	MAN-LMMRLP--ISLRS-----FVSASSSSNG-SPPVIGGSSGGVG
Barley	MASTSSLDLTKIVPPAGFLLHRRRTAVPSAPVLRGR--SKSGQCG--LAVSASSSNGAAGLFLPSDTEKKK
Rye	MASIPSLDLTKIVPPAGFLLHRRRAVSSAPVLWGK--GASGRCG--LAVSVLSSNGAAGLSPLSDSEKKK
Rice	MAASSSLDLVKTMAASGFLLR-CPAAPSAPVPLWGRS-GRGGGG--LAFSASSSNGAAVPSLSLSDSEKKK
Sorghum	MASSTLTLA--MGPG--VLVPRSAVPYASFARGRGRSASGRRSRNLVLSASFNGAAVPSLTTDSEKKK
Maize	MASSTLTLA--MGPG--ALLPRSAVPYASFAPGRGRSASGTRSRNLALSASSSNGAVVPSLTTDSEKKK
Human	-----MGL-----RAGGTLGRAGAGRGAPEGPGPSGGAQGGSIHSGRIA AVHNVPLSV---
Yeast	-----MSLQSN-SVKPTEIPLSE---
Tomato	PVILELPLDKIRRLMRTRSNDQQVKELMDSIKEIG-----LQVPIDVLE-VDGV-
Potato	PVILELPLDKIRRLMRTRSNDQQVKELMDSIKEIG-----LQVPIDVLE-VDGV-
<i>Arabidopsis</i>	PMIVELPLEKIRRLMRTRSNDQNKVKELMDSIRQIG-----LQVPIDVIE-VDGT-
Barley	PVVMEIPLDQIRRLMRTRANDPEKVQELMDSIRVIG-----LQVPIDVLE-VDGV-
Rye	PVVMEIILEDIRRLMRTRANDPKVQELMDSIRVIG-----LQVPIDVLE-VDGV-
Rice	PVVMEIPLDKIRRLMRTRANDPAKVQELMDSIRVIG-----LQVPIDVLE-VDGV-
Sorghum	PVIMEIPLDQIRRLMRTRANDPVKVQELMDSIRVIG-----LQVPIDVLE-VDGV-
Maize	PVIMEIPLDQIRRLMRTRANDPVKVQELMDSIRVIG-----LQVPIDVLE-VDGV-
Human	-----LIRPLPS--VLDPAKVQSLVDTIREDP-----DSVPPIDVL-WIKGAQ
Yeast	-----IRRPLAP--VLDPQKIDAMVATMKGIP TASKTCSLEQAEAAASAGELPPVDVL-GVRVKG
Tomato	---YGFSGCHRYEAHQQLGLLT-----IRCKIRRGTKETLRHHLR----- 124
Potato	---YGFSGCHRYEAHQQLGLPT-----IRCKIRRGTKETLRHHLR----- 124
<i>Arabidopsis</i>	---YGFSGCHRYEAHQQLGLPT-----IRCKIRKGTKETLRHHLR----- 125
Barley	---YGFSGCHRYEAHQQLGLPT-----IRCKVRRGTKETLRHHMR----- 154
Rye	---YGFSGCHRYEAHQQLGLPT-----IRCKVRRGTKETLRHHMR----- 154
Rice	---YGFSGCHRYEAHQQLGLPT-----IRCKVRRGTKETLRHHMR----- 154
Sorghum	---YGFSGCHRYEAHQQLGLPT-----IRCKVRRGTKETLRHHMR----- 154
Maize	---YGFSGCHRYEAHQQLGLPT-----IRCKVRRGTKETLRHHMR----- 154
Human	GGDYFYSGGCHRYAAYQQLQRET-----IPAKLVQSTLSDLRVYL-GASTPDLQ 137
Yeast	-QTLYYAFGGCHRLQAYDRRARETQNAAFPVRCRVLPATPRQIRMYL-GSSLDIE- 127

Figure 1 Comparison of the deduced amino acid sequences of Srx proteins from eight plant species, human and yeast (*S. cerevisiae*). The conserved signature sequence (FG/SGCHR) is boxed. The residues corresponding to Arg51 and Lys61 of hSrx (indicated by arrowheads) were also conserved in plant and yeast Srx proteins. The GenBank accession numbers for *Arabidopsis*, rice, human and yeast Srx sequences are BT004731, DQ132851, AAH47707 and P36077, respectively. The transcripts, from which the deduced amino acid sequences of the putative Srxs from barley, maize, potato, rye, sorghum and tomato are derived, were identified in TIGR Gene Indices website (<http://www.tigr.org/tdb/tgi/plant.shtml>). Their accession numbers are TC141756, TC250781, TC97423, TC2742, TC105843 and TC160927, respectively.

Prx proteins (A and B) in response to the oxidation stress imposed in this work were also investigated.

Characterization of *AtSrx* knock-out mutant

A T-DNA insertion line, SALK_015324, was identified for *AtSrx* and obtained from *Arabidopsis* Biological Resource Center (ABRC, <http://www.biosci.ohio-state.edu/>). Plants homozygous for T-DNA insertion were identified from T3 population and confirmed in the T4 generation using genomic PCR according to published methods (<http://signal.salk.edu/>). The lack of *AtSrx* transcripts in the homozygous T-DNA insertion line (designated as *atsrx1-1*) was confirmed using RT-PCR with *AtSrx* specific primers (Table 1).

For comparing the oxidation stress response of *atsrx1-1* and wild type plants, seedlings (grown under standard condition, with 6 to 9 rosette leaves) were transferred onto MS medium supplemented with 10 μ M paraquat (methyl viologen, Sigma). After growing for a further seven days (under 16 h light/8 h dark photoperiod), the phenotypes of the treated plants were recorded using a digital camera. *atsrx1-1* and wild type plants were also tested for difference in maximal quantum yield of photosystem II (F_v/F_m ratio), which is inversely related to the severity of stress [26, 27], before and after oxidation treatment using dark-adapted leaves as described previously [28]. For investigating maximal quantum yield before oxidation stress, the mature rosettes of *atsrx1-1* and wild type plants (4-week old, grown under standard condition) were transferred to dark. After 20 min in the dark, the fully expanded leaves were used for determining F_v/F_m ratio. For investigating maximal quantum yield after oxidation stress, the mature rosettes of *atsrx1-1* and wild type plants (as described above) were sprayed with a solution containing 100 mM paraquat and 0.5% Tween 20 (Sigma). The sprayed plants were kept in dark for 3 h (at 22 °C) before being transferred to standard growth condition (with 16 h light/ 8 h dark photoperiod). F_v/F_m ratio was determined (towards the end of the 8 h dark photoperiod) at 24 h and 48 h after shifting to the normal growth condition, respectively.

As an essential component to above genetic analysis, complementation experiment was conducted to examine if cloned *AtSrx* cDNA could correct the phenotype of *AtSrx* knock-out line under oxidation stress. For this purpose, a 35S-*AtSrx* expression cassette was created by cloning the coding sequence of *AtSrx* into pJIT163 (see above). This cassette was then ligated into the *Sac*I and *Sma*I sites of the plant transformation vector pBINPLUS [29], creating the T-DNA construct pBin35S-*AtSrx*. The T-DNA cassette was introduced into *atsrx1-1* background using *Agrobacterium* mediated transformation [30]. Kanamycin resistant T1 plants (with 6 to 9 rosette leaves) and wild type plants of similar developmental stage were transferred onto MS medium containing 10 μ M paraquat. The phenotypes of the treated plants were examined and recorded as described above.

Results

Primary structure of putative Srx proteins from higher plants

Prior to this work, the presence of a putative Srx homolog in *Arabidopsis* (*AtSrx*) has been noted [5]. However, there was no knowledge on Srx in other plant species. By searching public databases using *AtSrx* as query, potential Srx encoding sequences were found in a variety of plant and algae species. For investigating general features in

the primary structure of plant Srx proteins in this work, the deduced amino acid sequences of putative Srxs from five monocot (barley, Sorghum, rice, rye, maize) and three dicot (*Arabidopsis*, potato, tomato) species were analyzed in more detail. At the amino sequence level, plant Srxs were 21.6-25.0% identical to their yeast ortholog and 19.0-30.3% identical to hSrx. Among the eight plant Srxs, the highest identity score was 99.2% (between potato and tomato Srxs), whereas the lowest one was 58.9% (between tomato and rye Srxs). The signature sequence Phe-Gly/Ser-Gly-Cys-His-Arg was present in all eight plant Srxs, and the Gly-Cys-His-Arg element (within the signature sequence) was strictly conserved (Figure 1, boxed region). Apart from signature sequence, the two residues corresponding to Arg51 and Lys61 of hSrx were also strictly conserved in the eight plant Srxs (Figure 1, indicated by arrowhead). Using ChloroP program, the eight plant Srxs were all predicted to contain chloroplast transit peptide in the N-terminus of their primary structure (data not shown). A similar finding was also obtained when the TargetP program was employed for prediction.

Complementation of yeast Srx deficiency by *AtSrx* or *OsSrx*

In yeast, functional deficiency of Srx (by deleting the *SRX1* gene) renders mutant cells hypersensitive to oxidation stress elicited by exogenous oxidant (such as hydrogen peroxide) [5]. To examine if plant Srx would function intracellularly, the cDNAs of *Arabidopsis* and rice Srxs were chosen as representatives for complementing Srx deficiency

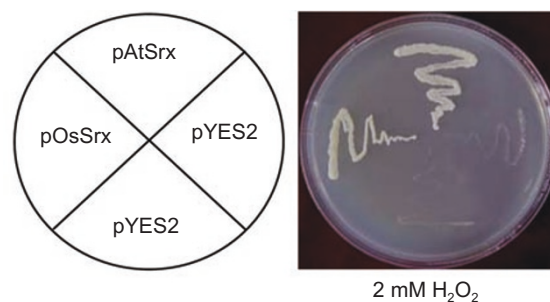


Figure 2 Complementation of functional deficiency of Srx in *SRX1* deletion yeast cells by Srx homolog from *Arabidopsis* and rice. Three DNA constructs, pAtSrx (for expressing *Arabidopsis* Srx), pOsSrx (for expressing rice Srx) and pYES2 (as vector control), were each transformed into *SRX1* deletion yeast strain. Recombinant cells were selected (using uracil free medium and PCR amplification of *AtSrx* or *OsSrx* coding sequences) and were further streaked onto plates containing 2% galactose and 2 mM hydrogen peroxide (H_2O_2). Functional complementation of yeast Srx deficiency by plant Srx homolog is indicated by restored growth of the recombinant *SRX1* deletion cells containing pAtSrx or pOsSrx in the presence of H_2O_2 .

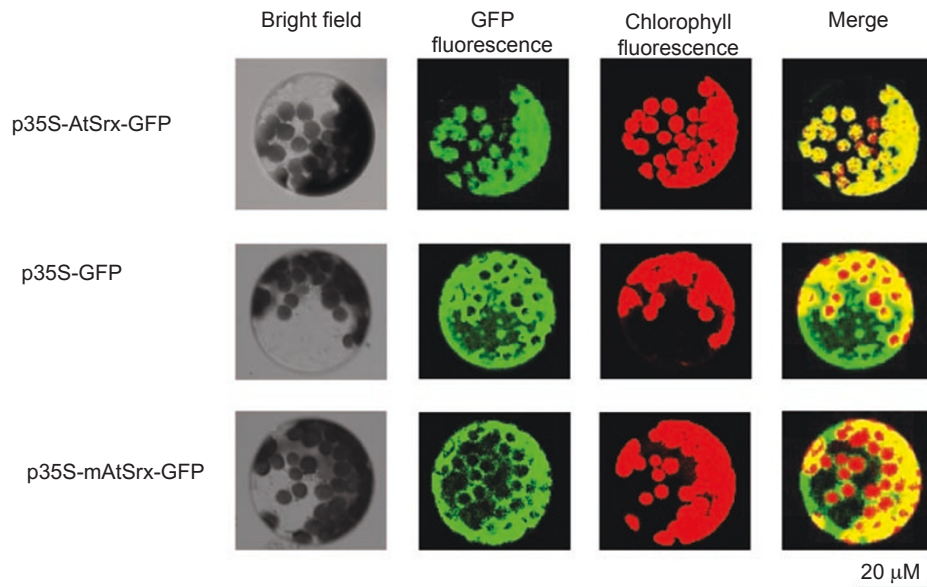


Figure 3 Intracellular targeting of AtSrx. *Arabidopsis* mesophyll protoplasts were transfected with p35S-AtSrx-GFP (expressing a wild type AtSrx-GFP fusion protein, top panel), p35S-GFP (expressing GFP alone, middle panel), or p35S-mAtSrx-GFP (expressing the GFP fusion protein of a truncated AtSrx lacking the first 30 amino acid residues that were predicted to form a chloroplast transit peptide, bottom panel). Expressing protoplasts were examined using dual channel confocal microscopy. Chloroplast targeting is demonstrated by colocalization of GFP fluorescence and chloroplast, and presence of chloroplast-associated yellow signal when green (GFP) and red (chloroplast) fluorescences were merged. Lack of chloroplast targeting is indicated by presence of GFP fluorescence in the cytoplasm surrounding chloroplasts, and the absence of chloroplast-associated yellow signal in the green (GFP) and red (chloroplast) fluorescence-merged image. Bar=20 μ M.

in *SRX1* deletion yeast cells. From Figure 2, it is clear that ectopic expression of AtSrx or OsSrx restored the growth of *SRX1* deletion yeast cells in the presence of hydrogen peroxide, indicating that plant Srx could complement functional deficiency of Srx in *SRX1* deletion yeast cells.

Targeting of AtSrx to chloroplast

The prediction of chloroplast transit peptide led us to test if plant Srx would be targeted to chloroplast *in vivo*. To this end, two *AtSrx-GFP* fusion constructs and one control construct expressing *GFP* coding sequence alone were transiently expressed in *Arabidopsis* mesophyll protoplasts, and the patterns of subcellular GFP fluorescence among the three sets of transient expressions were compared using confocal microscopy. In the protoplasts transfected with p35S-AtSrx-GFP, which expressed wild type *AtSrx-GFP* fusion cistron, the green fluorescence was clearly associated with chloroplasts and colocalized with the red autofluorescence of chloroplasts (Figure 3, top panel). In contrast, in the protoplasts transfected with the control construct p35S-GFP (expressing *GFP* coding sequence alone, Figure 3, middle panel) or p35S-mAtSrx-GFP (expressing a mutated *AtSrx-GFP* fusion cistron in which the coding sequence for the predicted chloroplast transit peptide in

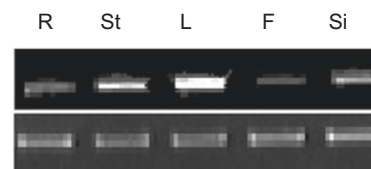


Figure 4 Evaluation of relative transcript levels of AtSrx in *Arabidopsis* root (R), stem (St), leaf (L), flower (F) and silique (Si) organs using semiquantitative RT-PCR. Amplification of tubulin transcripts was employed to normalize cDNA contents and to monitor the kinetics of thermoamplification during PCR. AtSrx transcript levels were highest in leaf, lowest in flower, and intermediate in other organs. Reproducible transcriptional patterns were obtained in independent assays.

AtSrx protein was deleted, Figure 3, bottom panel), the green fluorescence was distributed in the cytoplasm surrounding the chloroplasts and was not colocalized with the red autofluorescence of chloroplasts.

Transcriptional patterns of AtSrx in Arabidopsis organs and during stress

Using semiquantitative RT-PCR, the relative transcript

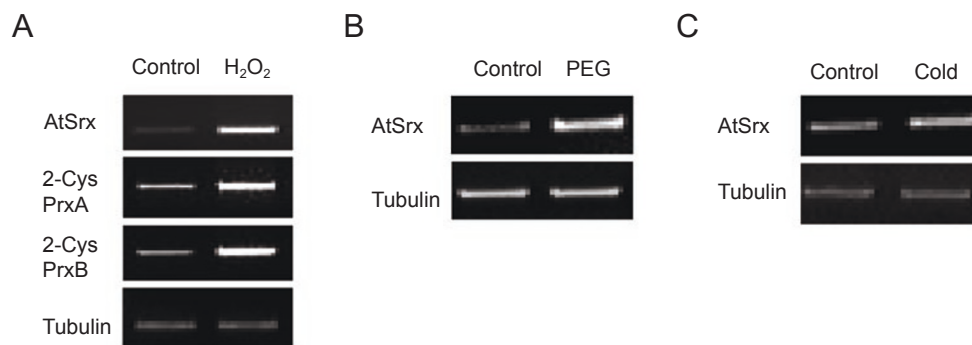


Figure 5 Changes of AtSrx transcript levels in response to oxidation (by H₂O₂, **A**), osmotic/water deficit (by PEG8000, **B**), and cold (by growing seedlings at 4 °C, **C**) stresses using semiquantitative RT-PCR. Amplification of tubulin transcripts was used for normalizing cDNA contents and monitoring thermoamplification kinetics. Alterations in the transcript levels of 2-Cys Prx proteins A and B in response to the oxidation stress imposed in this work were also investigated.

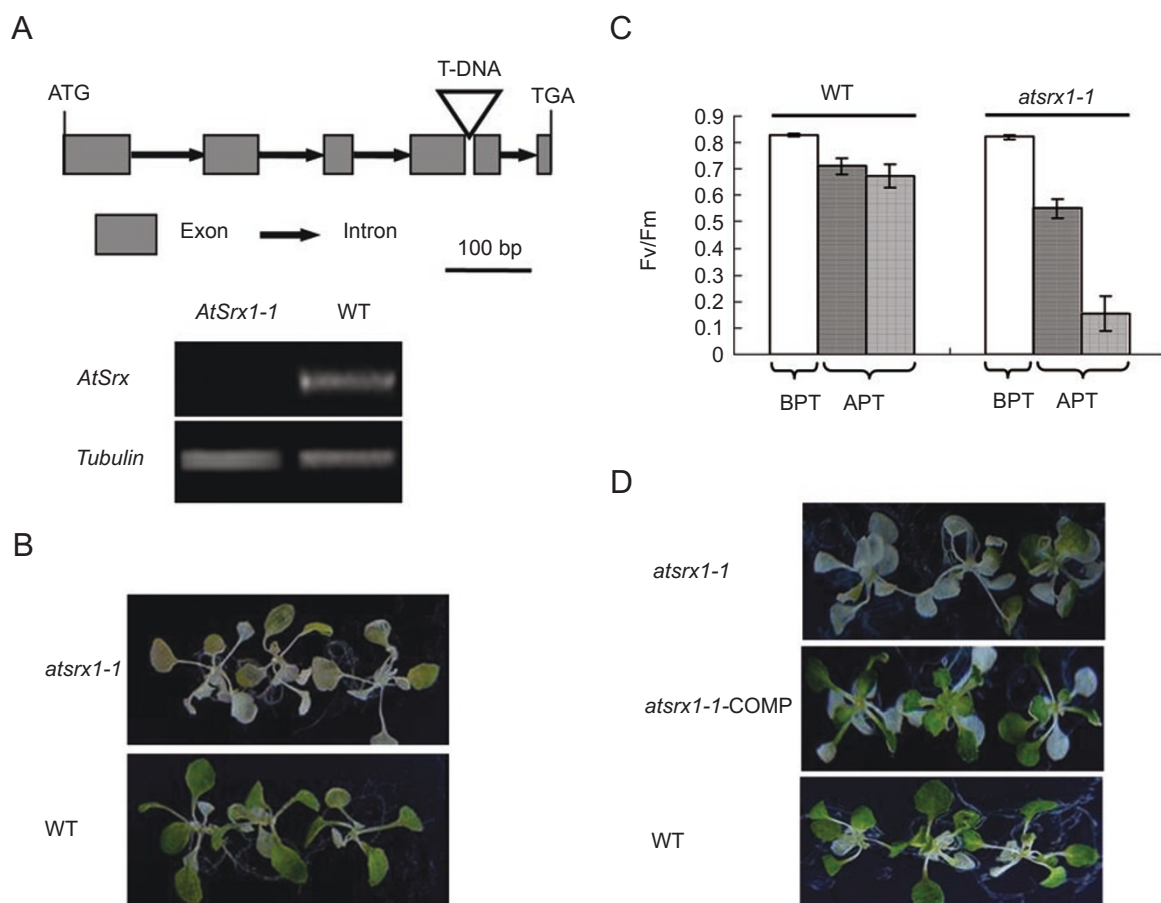


Figure 6 Characterization of *AtSrx* knock-out mutant (*atsrx1-1*) and its response to oxidation stress elicited by the herbicide paraquat. **(A)** A diagram showing the position of T-DNA insertion within the genomic open reading frame of *AtSrx* (top panel). *AtSrx* transcripts were detected in wild type (WT) but not *atsrx1-1* plants (bottom panel). **(B)** When grown on MS medium with 10 μM paraquat, the chlorosis phenotype of *atsrx1-1* was much more severe than that of WT plant. **(C)** *atsrx1-1* and wild type (WT) plants were similar in F_v/F_m ratio before paraquat treatment (BPT, white column). But at either 24 h (striped column) or 48 h (dotted column) after paraquat (100 μM) treatment (APT), the F_v/F_m ratio was more drastically decreased in *atsrx1-1* mutant than in WT plants. **(D)** In complementation experiment conducted using MS medium containing 10 μM paraquat, the phenotype of *atsrx1-1*-COMP T1 plants, in which *AtSrx* expression was restored by genetic transformation was, similar to that of WT plants. By contrast, the *atsrx1-1* plants displayed severe chlorosis in their leaves.

levels of AtSrx in *Arabidopsis* vegetative and reproductive organs were evaluated. AtSrx transcript levels were highest in leaves and intermediate in roots, stems and siliques (Figure 4). In contrast, AtSrx transcription was very low in flowers and required at least 35 amplification cycles to be detected (Figure 4). This transcriptional pattern was reproducibly obtained in multiple independent experiments.

The influence of oxidation stress (conferred by 4 mM hydrogen peroxide), osmotic/water deficit stress (mediated by 10% PEG8000), and cold treatment (8 h at 4 °C) on AtSrx transcript levels was also examined. The application of hydrogen peroxide substantially increased the transcript level of AtSrx as well as those of 2-Cys PrxA and 2-CysPrxB (Figure 5A). Osmotic/water deficit and cold treatments also raised AtSrx transcript level considerably (Figure 5B and 5C). The above transcriptional patterns were each tested in multiple experiments and were found to be reproducible in independent assays.

Response of AtSrx knock-out line (atsrx1-1) to oxidation stress

Further to above experiments, a T-DNA insertion mutant (*atsrx1-1*) deficient in AtSrx transcripts was identified (Figure 6A), and its response to oxidation stress was compared to that of wild type *Arabidopsis* plants in this work. Under standard growth condition, *atsrx1-1* and wild type plants were indistinguishable in morphology and development (data not shown). When grown on MS plate containing 10 μM paraquat, a herbicide with a well-known role in increasing ROS production and oxidation stress in chloroplast [31], the growth of *atsrx1-1* and that of wild type plants was both affected (Figure 6B). However, the leaf chlorosis phenotype displayed by *atsrx1-1* mutant was much more severe than that exhibited by wild type strain (Figure 6B). *atsrx1-1* and wild type plants did not differ significantly in F_v/F_m ratio before paraquat treatment (BPT, Figure 6C). However, the decrease of F_v/F_m ratio in the knock-out line (*atsrx1-1*) was significantly greater than that shown by wild type strain at either 24 h or 48 h after paraquat treatment (APT, Figure 6C). In complementation experiment, the phenotype of *atsrx1-1*-COMP plants, into which AtSrx cDNA had been reintroduced through *Agrobacterium* mediated transformation, was similar to that of wild type plants on paraquat containing medium (Figure 6D), indicating that the hypersensitive phenotype of *atsrx1-1* plants to paraquat treatment could be corrected by restored expression of AtSrx.

Discussion

In this work, four complementary lines of evidence were obtained for the existence of functional Srx protein

in both monocot and dicot plant species. First, the deduced amino acid sequences of putative Srxs from eight plant species exhibited significant identities to those of yeast and mammalian Srx proteins, and contained the conserved signature sequence and residues essential for catalysis. Second, ectopic expression of *Arabidopsis* or rice Srx coding sequence complemented Srx functional deficiency in *SRX1* deletion yeast cells. Third, the transcript level of AtSrx was substantially increased under oxidation stress imposed by hydrogen peroxide. Fourth, the knock-out line of *AtSrx*, *atsrx1-1*, was more susceptible to oxidation stress elicited by paraquat than wild type *Arabidopsis* plant. The strong conservation in the primary structure of plant, yeast and human Srxs indicates that there may be high similarity in the basic biochemical mechanism underlying reduction of protein sulfinic acid among eukaryotic organisms. Consequently, the knowledge on yeast and animal Srxs may aid biochemical and molecular analysis of plant Srxs in the future.

Because of the essential role of chloroplast in carbon assimilation and synthesis of amino acids, fatty acids and nucleotides, the redox state of chloroplast must be correctly maintained. In addition to CuZn-superoxide dismutase and ascorbate peroxidase that are known to play a prominent role in detoxifying ROS in chloroplast [32, 33], more recent investigations have shown that 2-Cys Prx proteins are also essential in peroxide removal during photosynthetic electron flux [11]. In this work, we have shown that an AtSrx-GFP fusion protein was targeted to chloroplast and that the transcript level of AtSrx was highest in photosynthetic leaves. AtSrx transcription could be substantially increased by oxidant and this increase paralleled with enhanced transcription of 2-Cys Prx proteins. Most importantly, based on the severity of the chlorosis phenotype and the changes in F_v/F_m ratio, which is a sensitive and reliable indicator of stress [26, 27], after paraquat treatment, knock-out of *AtSrx* expression rendered *Arabidopsis* plants much more susceptible (than the wild type control) to increased ROS production in chloroplast and this hypersensitivity was reversed by restored *AtSrx* expression through genetic transformation. Collectively, these data indicate that the expression and function of AtSrx in chloroplast is essential for proper plant response to oxidation stress conditions. Considering that 2-Cys Prx is an important component of antioxidant defense in chloroplast and there exists functional cooperation between 2-Cys Prx and Srx in yeast and mammalian cells [5, 6, 12], it is tempting to suggest that there may be functional cooperation between Srx and 2-Cys Prx in detoxifying peroxides in chloroplast. In this respect, it is interesting to note that 2-Cys Prx with its catalytic cysteine residue modified to sulfinic acid has been detected in chloroplast [34]. However, further experiments

are needed to examine if plant Srx would indeed interact with 2-Cys Prx and whether plant Srx can reduce Cys-SO₂H *in vitro* and *in vivo*. It will also be interesting to investigate in more depth if Srx (alone or together with 2-Cys Prx or other partners) would confer protection to chloroplast metabolism including photosynthesis and the synthesis of amino acids, fatty acids and nucleotides.

Because of their sessile life style, plants have evolved sophisticated mechanisms to respond to environmental stresses. A general feature of plant response to biotic and abiotic stresses is production of ROS [10]. Although stress generated ROS have recently been proposed to act as signaling molecules to facilitate plant adaptation to adverse environments [35], the underlying mechanism(s) is still not well understood. In yeast and mammalian cells, reversible oxidation of 2-Cys Prx, which involves the function of Srx, has been found essential in mediating hydrogen peroxide-induced gene expression changes [12, 36]. Interestingly, drought and cold stresses have been shown to increase the amount of 2-Cys Prx with its catalytic cysteine residue modified to Cys-SO₂H in barley [37], though it is still unknown if there would be any changes in the reduction of this over-oxidized 2-Cys Prx during stress. We have demonstrated in this work that osmotic/water deficit and cold stresses could substantially increase the transcript level of AtSrx, indicating that there may be enhanced reduction of protein sulfinic acid by Srx under these stress conditions. In line with this reasoning, signaling by ROS has been considered to be an integral part of plant responses to osmotic/water deficit and cold stresses [38]. The next challenges are to understand potential impact of enhanced Srx expression on reversible oxidation of 2-Cys Prx and its implication on ROS mediated signaling in plants under stress.

From above discussions, it might be understandable why Srx was found to be highly expressed in leaves and its transcripts were further increased under stresses. However, AtSrx transcripts were also detected in roots, flowers and siliques albeit at relatively lower levels. This suggests that the physiological function of Srx may be required in most plant tissues under normal growth condition, but its expression level in different tissues (and organs) is subject to transcriptional regulation. A recent study has revealed that the level of protein oxidation is drastically reduced during bolting and flowering in *Arabidopsis* [39]. This provides an important clue for further investigations into potential mechanism involved in transcriptional regulation of AtSrx during flower development.

In summary, this work has generated novel insights into the existence and potential functionality of Srx homolog in higher plants. Based on this and previous investigations, it is hypothesized that there may be functional interaction

between Srx and 2-Cys Prx proteins in plant cells. This interaction is likely to be important for maintaining redox balance in chloroplast and enabling plants to adapt to abiotic environmental stresses that involve ROS production. To advance this work, we are now in the process to analyze the biochemical mechanism and biological function of Srx in plant cells.

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