THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 28, Issue of July 9, pp. 19714–19722, 1999

Printed in U.S.A.

In Vivo Characterization of a Thioredoxin h Target Protein Defines a New Peroxiredoxin Family*

(Received for publication, February 24, 1999)

Lionel Verdoucq‡§, Florence Vignols‡, Jean-Pierre Jacquot¶, Yvette Chartier‡, and Yves Meyer‡‖

From the ‡Laboratoire de Physiologie et de Biologie Moléculaire des Plantes, UMR 5545, Université de Perpignan, Avenue de Villeneuve, F 66025, Perpignan, France and the ¶Laboratoire de Biologie Forestière, Associé INRA, Université de Nancy 1, BP 239, F 54506, Vandoeuvre, France

Disruption of the two thioredoxin genes in yeast dramatically affects cell viability and growth. Expression of *Arabidopsis* thioredoxin AtTRX3 in the *Saccharomyces* thioredoxin Δ strain EMY63 restores a wild-type cell cycle, the ability to grow on methionine sulfoxide, and H_2O_2 tolerance. In order to isolate thioredoxin targets related to these phenotypes, we prepared a C35S (*Escherichia coli* numbering) thioredoxin mutant to stabilize the intermediate disulfide bridged complex and we added a polyhistidine N-terminal extension in order to purify the complex rapidly. Expression of this mutant thioredoxin in the wild-type yeast induces a reduced tolerance to H_2O_2, but only limited change in the cell cycle and no change in methionine sulfoxide utilization. Expression in the Δ thioredoxin strain EMY63 allowed us to isolate a complex of the thioredoxin with YLR109, an abundant yeast protein related to PMP20, a peroxisomal protein of *Candida*. No function has so far been attributed to this protein or to the other numerous homologues described in plants, animals, fungi, and prokaryotes. On the basis of the complementation and of low similarity with peroxiredoxins, we produced YLR109 and one of its *Arabidopsis* homologues in *E. coli* to test their peroxiredoxins activity. We demonstrate that both recombinant proteins present a thioredoxin-dependent peroxidase activity *in vitro*. The possible functions of this new peroxiredoxin family are discussed.

Thioredoxins are small disulfide-containing redox proteins (≈13 kDa) that have been isolated from almost all organisms (reviewed in Refs. 1 and 2). Three types of function have regularly been proposed. In the first type, they act as structural components required for the activity and synthesis of some components of T7 DNA polymerase or for phage assembly. These reactions are mostly redox independent, while the two other types are directly redox dependent. In the second type, they are intermediate energy donors to some enzymes like ribonucleotide reductase, PAPS[1] reductase, methionine sulfoxide reductase, and hydrogen peroxide reductase which accept energy through a proton transfer on cysteines. In the third type, they regulate the function of enzymes or transcription factors by modifying their disulfide bridged conformation. Examples of redox regulated enzymes are most Calvin cycle enzymes, CF1 and malate dehydrogenase of plant chloroplasts (3). Redox regulated transcription factors have been described in mammals, for example, NF-κB and AP1 (4). In most studies, the suggestion that a thioredoxin is the cellular reductant of a particular protein is sustained almost exclusively by *in vitro* experiments. Nevertheless, other cellular reducers like glutaredoxins, NADPH reductases, or even glutathione are also able to reduce disulfide bridges. The situation is even more complex in plants which present multiple thioredoxin genes and their products. For example, the *Arabidopsis thaliana* genome encodes at least five cytosolic thioredoxins h (5), and seven chloroplastic thioredoxins (2). In addition, other proteins with thioredoxin domains have been described in plants (6–10).

Mutants are useful tools for the characterization of gene function. Budding yeast presents two thioredoxin genes. While the inactivation of each of the genes does not significantly alter yeast growth, the disruption of both genes profoundly affects cell viability. This mutant strain (EMY63) is unable to use sulfate as sole a sulfur source and grows very poorly on methionine sulfoxide. Rapid growth is obtained with methionine but the cell cycle is profoundly modified, with a longer S phase and a shorter G_1 phase. Moreover, this mutant yeast shows an increased sensitivity to hydrogen peroxide (11, 12). We have previously shown that the five *A. thaliana* thioredoxins h (AtTRX1 to AtTRX5) confer a normal cell cycle and the ability to grow on methionine sulfoxide as unique sulfur source when expressed in the yeast mutant. AtTRX3 confers H_2O_2 tolerance but cannot restore sulfur assimilation, while AtTRX2 restores sulfur assimilation, but is unable to confer H_2O_2 tolerance (13). These data clearly indicate that yeast thioredoxins interact with multiple targets while each *A. thaliana* thioredoxin h interacts only with some of them.

One way to characterize the exact function of the unique cytosolic Trx in mammals or of each Trx in plants would be the isolation of *in vivo* complexes between one thioredoxin and its target(s) protein(s). This approach has been unsuccessful so far, probably because the complexes have a very short half-life. At the present time, only two articles, one on human, the second on plants report indirect evidence for such complexes using the two-hybrid system (14, 15). In this paper, we have developed a new *in vivo* approach in order to isolate biochemically complexes between thioredoxins and their cellular targets. We have used the recent knowledge on the reaction mechanism of thioredoxins to stabilize the complexes.

* This work was supported in part by Groupement de Recherche et d'Etude sur les Génomes/CNRS grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a Ph.D. fellowship from the French "Ministère de la Recherche et de la Technologie."

‖ To whom correspondence should be addressed. Tel.: 33-4-68-66-22-25; Fax: 33-4-68-66-84-99; E-mail: ymeyer@univ-perp.fr.

[1] The abbreviations used are: PAPS, adenosine 3′-phosphhate 5′-phosphosulfate; Trx, thioredoxin; YNB, yeast nitrogen base; ORF, open reading frame; PCR, polymerase chain reaction; DTT, dithiothreitol; TPx, Trx peroxidase; NTR, NADPH Trx reductase; PAGE, polyacrylamide gel electrophoresis; EST, expressed sequenced tag; TSA, thiol-specific antioxidant; Prx, peroxiredoxin; Grx, glutaredoxin; PAGE, polyacrylamide gel electrophoresis.

In vitro studies have shown that Trx reduce protein disulfide bridges through a two-step reaction involving the two cysteine residues of the conserved redox active site WCxPC (x = G or P). In the first step, the more N-terminal cysteine of the Trx (equivalent to the Cys^{32} of *Escherichia coli* TrxA) attacks the target protein disulfide bridge, reducing one cysteine of the S-S bridge and establishing a disulfide bridge with the second cysteine of the target, forming a mixed intermediate between the Trx and the target protein. The second step involves an intramolecular attack by the second cysteine of the Trx (equivalent to the Cys^{35} for the *E. coli* thioredoxin) on the mixed disulfide intermediate, releasing the reduced target protein and the oxidized Trx (16, 17). The intermediate disulfide bridged complex formed *in vitro* is stable if the second cysteine of the Trx is replaced by a structural analog of cysteine, like a serine or an alanine (18, 17).

In our report, we show that the ectopic expression of the C35S mutated AtTRX3 (amino acid numbering according to the *E. coli* thioredoxin) in *Saccharomyces cerevisiae* induces H_2O_2 hypersensitivity, thus partially mimicking the phenotype of the yeast Δ Trx mutant. This suggests that the dominant negative mutant protein undergoes a stable interaction with the target responsible for H_2O_2 tolerance. We have purified this complex and shown that it results from the interaction of the mutated Trx with the product of ORF YLR109, an abundant protein to which no function has so far been attributed. We show that the *E. coli* recombinant *YLR109* product presents all the characteristics of the peroxiredoxin family, and that its peroxidase activity is dependent of Trx activity. Moreover, we describe well conserved sequences similar to *YLR109* for almost all organisms, including mammals, plants, and bacteria, suggesting an identical peroxiredoxin activity for these proteins.

EXPERIMENTAL PROCEDURES

Plasmids, Strains, and Media—PCR products were cloned into the plasmid pMosBlue (Amersham Life Science), and introduced in *E. coli* strain DH5α. Plasmid pET16b (Novagen) was used to express recombinant protein in *E. coli* BL21(DE3), and the centromeric shuttle plasmid YCp2 (19) was used to express thioredoxin in *S. cerevisiae*. EMY60 is the standard wild-type strain (*Mat*a, *ade*2-1, *ade*3-100, *his*3-11, *leu*2-3, *lys*2-801, *trp*1-1, *ura*3-1) and EMY63 is the Trx double mutant, isogenic with EMY60 except at the Trx loci (*Mat*a, *ade*2-1, *ade*3-100, *his*3-11, *leu*2-3, *lys*2-801, *trp*1-1, *ura*3-1, *trx1::TRP1*, *trx2::LEU2*). These two strains were generous gifts from E. G. D. Muller (University of Washington). Yeast minimal medium YNBRaf contained 7 g $liter^{-1}$ Bacto-YNB (Difco) and 2% raffinose. YNBRGal minimal medium contained 7 g $liter^{-1}$ Bacto-YNB, 1% raffinose, and 2% galactose.

General Methods—Manipulation of DNA, *E. coli,* and *S. cerevisiae* were performed using standard methods (20, 21). *S. cerevisiae* cells were transformed after lithium chloride treatment (22). DNA synthesis by the polymerase chain reaction (PCR) was performed with the Pfu DNA Polymerase (Stratagene). DNA was purified with Qiaquick Gel extraction kit (Qiagen). All restriction enzymes were provided by Roche Molecular Biochemicals and T4 DNA ligase was provided by Promega. All constructs were checked by sequencing. Dideoxynucleotide sequencing was performed with the ABI Prism Big Dye Terminator Cycle Reaction Kit (Applied Biosystems), in our laboratory on a ABI 377A sequencer.

Mutagenesis of Trx Active Site—AtTRX3 and YTRX1 ORFs were amplified by PCR using specific oligonucleotides designed to introduce a *Nde*I site upstream of the start codon and a *Bam*HI site downstream from the termination codon. Site-directed mutagenesis of the thioredoxins active site were obtained by PCR-mediated overlap extension using a pair of complementary mutated oligonucleotides (23). Single amino acid mutants of Trx were created by PCR using the following nucleotide pairs: for C35S AtTRX3, oligonucleotide AtTrx3CSdir 3′ (5′-GCAACATGGTGCCCACCTTCACG-3′) and oligonucleotide AtTrx3CSdir5′ (5′-AAACGTGAAGGTGGGCACCATG-3′); for C35S YTRX1, oligonucleotide YTrx1CSdir5′ (5′- GCAATCATTTTACTTGGACCGCACC-3′) and oligonucleotide YTrx1CSdir3′ (5′-GGTGCGGTCCAAGTAAAATGATTGC-3′) and then cloned in pMosBlue plasmid. After dideoxynucleotide sequencing, the mutated DNA fragments were digested by *Nde*I and *Bam*HI restriction enzymes and subcloned into pET16b plasmid. This plasmid allows the fusion of a 10-His residue extension at the N-terminal part of the mutated thioredoxins, allowing purification on Ni^{2+} column (see below). A second round of PCR amplification was done with an oligonucleotide (16bMluIHis, 5′-TTTACGCGTAAGAAGGAGATATACCATGGGC-3′) designed to introduce a *MluI* restriction site upstream from the start codon of the polyhistidine extension, and with the same *Bam*HI site containing oligonucleotide. To express thioredoxins and thioredoxin mutants in yeast, we cloned the corresponding ORFs by using the two unique cloning sites *Mlu*I and *Bam*HI, present in the shuttle vector YCp2. This plasmid contains the *URA3* gene as a selectable marker and ensures protein production after induction of the *GAL1* promoter. Dideoxynucleotide sequencing was performed with the YCp5′ oligonucleotide (5′-CCTCTATACTTTAACGTCAAGG-3′), upstream from the *Mlu*I site in YCp2 plasmid.

Expression and Purification of Recombinant Thioredoxins on Ni^{2+} Column—Recombinant site-mutated Trx were purified from *E. coli* and *S. cerevisiae* cells. Expression conditions are different for *E. coli* and *S. cerevisiae* but purification procedures are the same. BL21(DE3) containing pET16b-mutated Trx were grown in 1 liter of LB medium up to $OD_{600\ nm} = 0.5$, and recombinant protein expression was induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Cells were pelleted and stored at −80 °C for the subsequent protein extraction. 10-ml cultures of EMY60 or EMY63, containing YCp-polyHis-mutated Trx, were grown at 30 °C in YNBRaf appropriate medium, and then diluted in 5 liters of YNBRGal medium with an initial $OD_{600\ nm} = 0.05$. Cells were collected at $OD_{600\ nm} = 1$, and stored at −80 °C. *E. coli*- or *S. cerevisiae*-induced cells were broken by a hydraulic press (Carver, model 3968) at 3500 p.s.i., at −80 °C. Broken cells were resuspended in 10 ml of chilled 1 × Binding Buffer (His.Bind™ System, Novagen), containing a protease inhibitor mixture (Complete™ Mini, EDTA-Free, Roche Molecular Biochemicals) and DNase A. Soluble proteins were recovered by centrifugation at 15,000 × *g* for 15 min. Cell fragments were frozen and submitted two more times to protein extraction. All protein-containing supernatants were pooled and applied on the His.Bind™ (Ni^{2+} resin) equilibrated column, and proteins were purified as recommended by Novagen. Purified proteins were washed with 50 mM Tris-HCl, pH 7.5, by ultrafiltration on a Microcon column (Amicon-Millipore) and submitted to subsequent analyses.

Enzymatic Activities—His-tagged YTRX1 thioredoxin activity was tested in a 500-μl reaction volume using the insulin-disulfide reduction assay (24). YTRX1 served as positive control and bovine serum albumin as a negative control. The assay was monitored by addition of 1 mM DTT, and measurements were performed at $OD_{650\ nm}$ for 45 min on a spectrophotometer (Model DU7400, Beckman). Metal-catalyzed oxidation DNA cleavage protection assays were performed as described previously (25) with the following modifications. Reactive oxygen species were generated for 30 min at room temperature by addition of 0.32 mM DTT in the presence of 3 μM $FeCl_3$. Reactions mixtures were incubated at room temperature with 20 μM YLR109 and 1 μg of plasmid DNA for 4 h. DNA degradation was checked by electrophoresis. His-tagged YLR109 and AtTPX2 peroxidase activity assays were performed as follows: the reaction was initiated by the addition of either *A. thaliana* NADPH Trx reductase (NTR: 180 nM) or H_2O_2 (100 μM) to 1 ml of 30 mM Tris-HCl, pH 8, reaction medium containing, 670 nM *Chlamydomonas reinhardtii* Trx h, 200 μM NADPH, 10–50 μg (0.5–2.5 μM) of Prx. The reaction was followed spectrophotometrically at 340 nm at 20 °C.

Immunoblotting—Purified proteins were submitted to denaturing SDS-PAGE on a standard 15% polyacrylamide gel (20), under reducing or nonreducing conditions. Gels were subsequently electroblotted for 40 min onto a nitrocellulose membrane. Rabbit polyclonal antibodies against YTRX1 and AtTRX3 (1:10000 dilution), goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad), and a colorimetric detection system (HRP color, Bio-Rad) were used to visualize protein and complexes according to the manufacturer's instructions.

Two-dimensional SDS-PAGE—Purified proteins were diluted in the standard SDS buffer without β-mercaptoethanol and submitted to a first SDS-PAGE on a 12% polyacrylamide gel. Gel slices were treated for 1 h with the reducing SDS buffer (containing 5% β-mercaptoethanol), applied on the top of 12% polyacrylamide gels and submitted to a second SDS-PAGE. Proteins were stained by Coomassie Blue, or revealed by silver nitrate detection, using the Bio-Rad Silver Stain Plus kit.

H_2O_2 Sensitivity and Flow Cytometry—To test H_2O_2 sensitivity, the transformed cells were first grown in YNBRGal up to a concentration of 10^7 cells per ml, and then diluted to $OD_{600\ nm} = 0.2$. Four 1/5 dilutions of this cell suspensions were prepared and a 15-μl droplet of each were

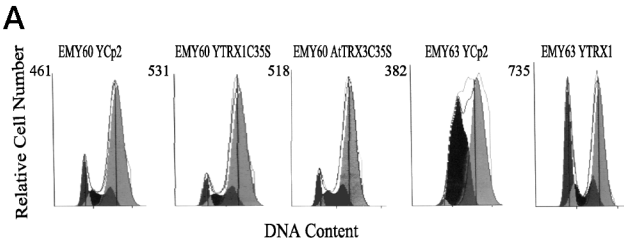
plated on YNBRGal-agar medium containing different H_2O_2 concentrations. Plates were then incubated 3 days at 30 °C. DNA content of the transformant cells was measured by a flow cytometry technique. 10 ml of cells were grown in YNBRGal medium to $OD_{600\ nm} = 0.5$, centrifuged, and washed with 10 ml of Tris-HCl, 50 mM, pH 8. Cells were fixed overnight at 4 °C in 70% ethanol, centrifuged, and resuspended in 1 ml of Tris-HCl, 50 mM, pH 8, containing 1 mg ml^{-1} RNase A, and incubated for 2 h at 37 °C. After centrifugation, the cell pellet was treated for 1 h at 50 °C with 50 μl of proteinase K at 10 $\mu g\ ml^{-1}$, resuspended in 500 μl of propidium iodide solution (50 $\mu g\ ml^{-1}$), and stained overnight in the dark at 4 °C. Fluorescence was analyzed using a Bruker ACR 1000 flow cytometer. Data were collected with the FRIED software which was used to estimate the proportion of cells in G_1 phase.

RESULTS

N-terminal His-tagged Yeast Trx 1 Is Fully Active in Vitro and in Vivo—Because our goal was the isolation of Trx targets and because Trx are not abundant proteins, we chose to use polyhistidine-tagged Trx in order to facilitate protein and complex purification. The first step of our work was to demonstrate that the addition of such His-extension does not modify thioredoxin activity *in vivo*. For this control, we chose the YTRX1 thioredoxin, which can restore wild-type phenotype when expressed in the Trx double mutant yeast EMY63. The *YTRX1* open reading frame was then introduced into a production plasmid, pET16b, using the *Nde*I/*Bam*HI restriction sites in order to fuse a polyhistidine extension at the N-terminal end. The recombinant protein was then produced in *E. coli* and purified on an Ni^{2+} column. The *in vitro* activity of thioredoxin is usually tested by measurement of insulin reduction and the subsequent β-chain precipitation is followed spectrophotometrically at 650 nm (24). We checked that YTRX1 sharing an N-terminal polyhistidine extension is fully active in the reduction of human insulin (data not shown). We also had to check that the His-tagged YTRX1 is still able to complement EMY63 phenotype. For this purpose, the His-tagged ORF was transferred into the centromeric shuttle plasmid YCp2 under control of the inductive *GAL1* promoter. Transformed yeast EMY63 (Δ YTRX1, Δ YTRX2) were selected on YNBRaf minimal medium, and then transferred to the YNBRGal medium to ensure induction (see "Experimental Procedures"). The His-tagged YTRX1 complements EMY63 in all aspects, allowing growth on sulfate or methionine sulfoxide, re-establishing a normal cell cycle and H_2O_2 tolerance (not shown).

HisYTRX1C35S and HisAtTRX3C35S Induce a Partial Dominant Negative Phenotype in S. cerevisiae—We have constructed two shuttle plasmids, one encoding the HisYTRX1C35S mutant and the second encoding HisAtTRX3C35S, a His-tagged and mutated version of *A. thaliana* AtTRX3. Expression of the wild-type AtTRX3 allows EMY63 to grow with a normal cell cycle on methionine and to use methionine sulfoxide efficiently as sole sulfur source. AtTRX3 also allows EMY63 to tolerate 0.8 mM H_2O_2, but does not allow growth on sulfate. Based on the catalytic mechanism of Trx, if the mixed disulfide intermediates are stable, C35S Trx mutants should compete with the endogenous Trx for the target proteins and induce a mutant phenotype in the wild-type yeast.

After introduction and induction of the mutant Trx in the wild-type yeast EMY60, the cells remained able to grow on sulfate and on methionine sulfoxide as sole sulfur source. This suggested that the mutated proteins do not efficiently compete with the endogenous Trx for the target proteins implicated in these aspects of the phenotype (not shown). In contrast, a small reduction of the number of cells in the G_1 phase is observed in cells expressing HisYTRX1C35S and HisAtTRX3C35S, compared with EMY60 YCp2 wild-type cells (Fig. 1). These results suggest a weak interaction between these mutated Trx and a protein involved in the S phase. But the most important phenotypic effect is a reduction of H_2O_2 tolerance induced by



B

Transformant cells	EMY60 YCp2	EMY60 YTRX1C35S	EMY60 AtTRX3C35S	EMY63 YCp2	EMY63 YTRX1
Percentage of cells in G1 phase (%)	11.3	7.8	8.4	1.2	29

FIG. 1. **DNA histograms of asynchronous transformed cultures of wild-type yeast (EMY60 YCp2), or y*trx1*, *ytrx2* mutant cells (EMY63 YCp2).** Cells were grown in YNBRGal medium to exponential phase and then prepared for flow cytometry as described under "Experimental Procedures." *A,* relative fluorescent intensity is plotted against the relative cell number. The two peaks represent 1n and 2n DNA content. *B,* proportion of cells in G_1 phase estimated by FRIED software analysis.

HisAtTRX3C35S as is shown by the reduced growth of EMY60 expressing HisAtTRX3C35S on 0.5 mM H_2O_2 (Fig. 2*B*). EMY60 expressing HisAtTRX3C35S was almost unable to grow on a medium containing 0.8 mM H_2O_2 (Fig. 2*C*). This strongly suggests that HisAtTRX3C35S competes efficiently with the endogenous yeast Trx, probably by the formation of a stable complex with the protein target(s) implicated in H_2O_2 tolerance. In contrast, expression of HisYTRX1C35S does not modify H_2O_2 tolerance of EMY60 cells (Fig. 2, *B* and *C*), because of a weak production of HisYTRX1C35S recombinant protein (data not shown).

Purification and Characterization of Mixed Disulfide Intermediates—In order to isolate the disulfide intermediate, cellular extracts of EMY60 and EMY63 yeasts expressing AtTRX3C35S were purified on Ni^{2+} column, under nondenaturing, nonreducing conditions (26). Purified extracts were then submitted to denaturing SDS-PAGE under nonreducing (without β-mercaptoethanol) conditions to preserve disulfide-bonded complexes, and analyzed by Western blot with anti-AtTRX3 antibodies (Fig. 3). For comparison, extracts of *E. coli* expressing HisAtTRX3C35S were purified on Ni^{2+} column and analyzed in the same way. In *E. coli,* most HisAtTRX3C35S is present as dimers and trimers (Fig. 3*A*). Although dimers could result from a disulfide bridge between C32 of the two monomers, the presence of trimers suggests that the additional cysteine situated in the N-terminal part of the protein is also implicated in disulfide bridges. In EMY60, most HisAtTRX3C35S is monomeric and some dimers are present. Only one faint band that could correspond to a complex is detectable at 50 kDa. In EMY63 (the Trx minus strain), although HisAtTRX3C35S is expressed at equivalent levels as in EMY60, a far more complex pattern is observed. Using anti-AtTRX3 antibodies, five abundant bands are detectable by Western blot. A two-dimensional electrophoresis consisting in a nonreducing SDS-PAGE as first dimension followed by a reducing SDS-PAGE was analyzed by silver staining (Fig. 3*B*). Two bands of the first dimension correspond to the Trx monomer and dimer, while three bands (molecular mass 34, 36, and 50 kDa) are composed of Trx associated with one protein of 20 kDa. The silver staining also reveals the presence, in the Ni^{2+} column eluate, of a free amount of the 20-kDa protein which is not disulfide-bonded to a Trx and which was consequently not detected by immunoblotting of the first dimension. One possibility is that the mutated Trx is bridged with one subunit of a

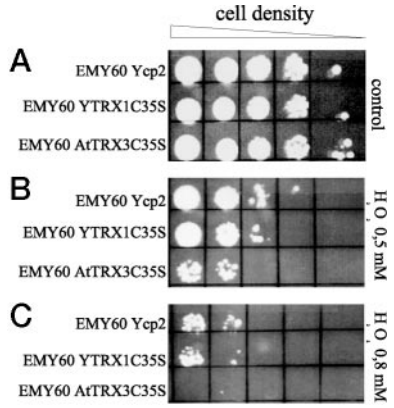


FIG. 2. **H_2O_2 tolerance of wild-type yeast EMY60 expressing mutated thioredoxins.** Cells were first grown in YNBRGal medium to a density of about 10^7 cells per ml, and then diluted to $OD_{600\ nm} = 0.2$. 1/5 dilutions were prepared and 15 μl of each were plated on YNBRGal medium containing different H_2O_2 concentrations, and incubated 3 days at 30 °C. *A,* control plate for cells growing without H_2O_2. *B,* plate containing 0.5 mM H_2O_2. *C*, plate containing 0.8 mM H_2O_2.

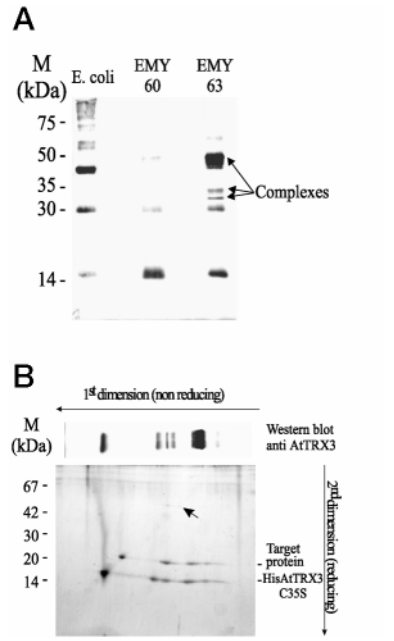


FIG. 3. **Interaction of HisAtTRX3C35S with target protein.** *A,* cellular extracts, expressing HisAtTRX3C35S, purified on Ni^{2+} column, and immunoblotted with anti-AtTRX3 antibodies after nonreducing SDS-PAGE. *B,* purified EMY63 HisAtTRX3C35S extracts submitted to two-dimensional PAGE. The first dimension is a nonreducing SDS-PAGE, and gel slices were submitted to a reducing second SDS-PAGE. The gel was stained with AgNi, and the protein panel is compared with immunoblot results. Positions of molecular size standards are indicated in kilodaltons (*lane M*).

target dimer, stabilized by noncovalent interactions. In the presence of SDS, the dimeric structure is destroyed, releasing a free target monomer and a Trx-target complex. In addition, a faint band of 40 kDa shown by an *arrow* is detectable. This band is abundant in large scale purifications performed using EMY63 and EMY60 cells and corresponds to alcohol dehydrogenase I (ADHI P00330), a Zn^{2+}-binding protein, and which shows affinity for Ni^{2+}. This protein is not related to Trx. In contrast, the 20-kDa band is present only in EMY63 cells expressing the mutated Trx HisAtTRX3C35S.

The Target Protein YLR109 Is Related to Abundant Prokaryotic and Eukaryotic Proteins—The 20-kDa protein band was purified from 1 liter of EMY63/HisAtTRX3C35S cells in exponential growth ($OD_{600\ nm} = 0.5$) on Ni^{2+} column followed by a reducing SDS-PAGE. The Coomassie Blue-stained band was submitted to microsequencing. Because the N-terminal part of the protein was blocked, trypsin peptides were analyzed and showed the following sequences: ETNPGTDVTVSSVE, MEV(DV)Q(VA)I(VI)K, DQVI(VA)VTXDNPYA, IGFELAVG-DGVYXS, A(NY)(PI)(VQ)Q(IV)(TG)(SF)N(AM)FQA, and (FM)-P(GQ)TVYPDV. All these sequences are present in the yeast ORF product YLR109, a predicted 19.1-kDa cytosolic protein of unknown function.

Similarities with other proteins were searched using blastx in the NCBI nonredundant data base. It appears that YLR109 is highly related to a peroxisomal membrane protein from *Candida boidini* (27) and to a small number of proteins from very different sources including prokaryotes (*Synechocystis, Hemophilus,* and *Rhyzobium*) and fungi (*Aspergillus, Malassezia,* and *Lipomyces*). We have searched for similar sequences in EST data bases using tblastn. Thirty-one human, 75 mouse, and 5 *Drosophila* ESTs allowed the reconstruction of a unique and complete cDNA for each organism. In higher plants, 32 *A. thaliana* ESTs were found which can be build in to two contigs. We have fully sequenced one complete clone of each contig corresponding to two highly related cDNAs, deposited under the names AtTPX1 (GenBank AF121355) and AtTPX2 (GenBank AF121356). One complete cDNA was also reconstituted from ESTs for rice and *Populus* and partial clones can be deduced from maize, *Pinus,* and *Ricinus* ESTs. This suggests the presence of highly conserved genes in fungi, animals, plants, and bacteria.

The multiple alignment shown in Fig. 4 indicates that the conservation is particularly high around Cys^{62} of YLR109. The good conservation of the C-terminal part of the proteins for all eukaryotic members is obvious. This part of the sequence addresses *C. boidini* PMP20 to the peroxisome, suggesting that in eukaryotes, all these proteins could be located in the peroxisome. In contrast, the prokaryotic sequences present various C termini. The *Hemophilus* sequence shows an interesting particularity: it is composed of two domains, the N-terminal region, which is similar to YLR109, followed by a C-terminal domain similar to a glutaredoxin active site. This structure reinforces the idea that all these proteins interact with dithiol reducers.

No function has been attributed to YLR109 and homologous proteins so far, but they are abundantly accumulated in *Saccharomyces* and in *Arabidopsis* callus as shown by the size of the spots on the proteomes publicly available at http://www.proteome.com/graph1.html for *Saccharomyces cerevisiae* and http://www.rs.noda.sut.ac.jp/~kamom/2de/2dacallus.html for *A. thaliana*. The N-terminal sequence of Spot PA0022 (hypothetical protein QA100011) exactly matches the AtTPX1 sequence. YLR109 have also recently been characterized to be three times more abundant after H_2O_2 treatment of *S. cerevisiae* cells (28).

YLR109 Shares Similarities with Thioredoxin-dependent Peroxidases—We further compared the YLR109 sequence to other proteins sharing weaker similarities. We found that YLR109 shares some similarity with other proteins characterized as thioredoxin-dependent peroxidases (TPx), also called TSA (thiol-specific antioxidant). The sequences around the putative catalytic active site of these proteins are more conserved. Cysteine 62 in YLR109 is always aligned with a conserved cysteine in the active site of these different thioredoxin-dependent peroxidases. We have constructed a phylogenic tree using DARWIN (29) with most TPx-related proteins (Fig. 5), including TSA homologues, alkyl hydroperoxide reductases, bacterioferritin comigratory proteins, 1-Cys peroxiredoxins (Prx), and other TPx homologues that have not been classified up to now.

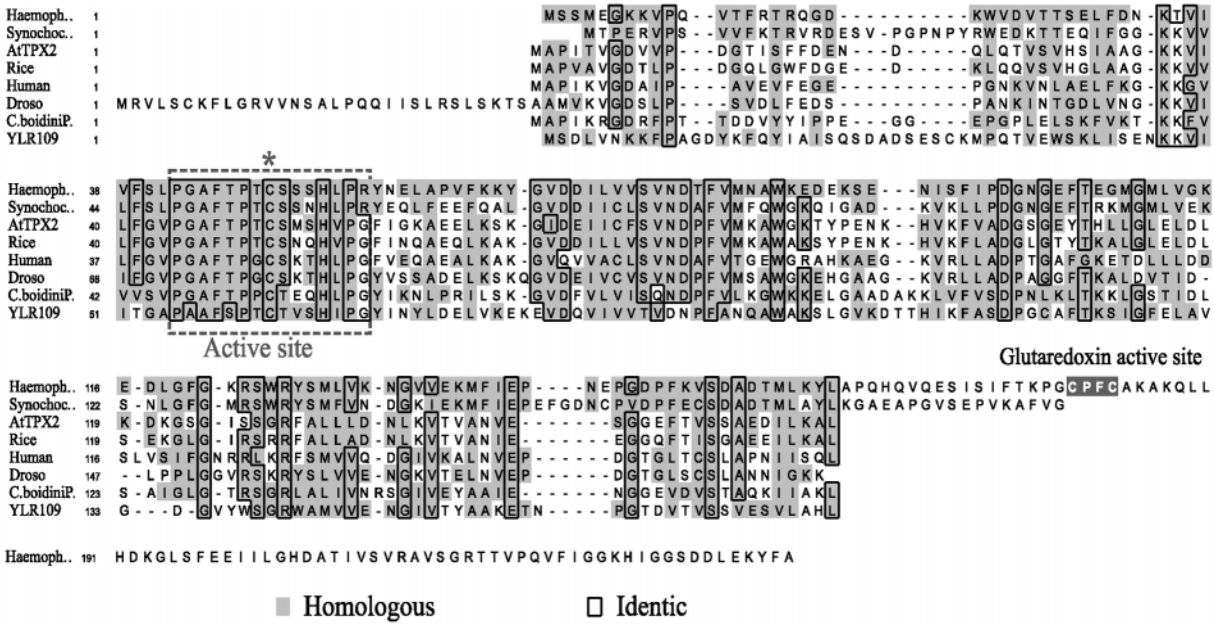


FIG. 4. **Multiple sequence alignment of YLR109-like proteins.** Deduced amino acid sequences of bacteria (*Hemophilus* U32739, *Synechocystis* D90909), plants (*Arabidopsis* AtTPX2 AF121356, Rice EST contig translation), animals (human and *Drosophila* EST contig translations), and fungi (*C. boidini* PMP20a P14292, *S. cerevisiae* YLR109 U53878) were aligned with ClustalW program (57), and presented SeqVu. Homologous residues are identified by *shaded areas*, and identical residues by *boxed areas*. The conserved cysteine is indicated by a *star* and the glutaredoxin active site in the *Hemophilus* sequence is *underlined*.

The tree clearly shows four distinct clusters (Fig. 5). Cluster 1 includes all classical 2-Cys TSA first discovered in yeast (30), the mammal TPx including four human sequences, the nuclear encoded chloroplastic Bas proteins from higher plants, which are close to the chloroplast encoded Bas of the red alga Porphyra and to the prokaryotic homologue of the blue alga *Synechocystis*. Numerous sequences from prokaryotes belong to this cluster: a subcluster associates bacterial alkyl hydroperoxide reductases, redox-dependent peroxidases which are reduced directly by a NADPH-dependent reductase, without a thioredoxin intermediate. Cluster 2 associates a subgroup of archebacterial sequences with another subgroup formed of 1-Cys peroxiredoxins (31). The sequences include rehydrins, a particular set of cytosolic proteins from plant seeds, and other human, yeast, and *Synechocystis* sequences. Cluster 3 is composed of sequences from prokaryotes and fungi. No biochemical information is available for these sequences which are described as bacterioferritin-associated proteins. Cluster 4 associated sequences similar to YLR109 as described previously. Sequences from fungi, animals, plants, and prokaryotes are members of this group.

YLR109 Displays Peroxidase Activity in Vivo—An important step in our work was to respond to the hypothesis that YLR109 and homologues belong to a new peroxidase family. We decided to test the ability of YLR109 to ensure a protection against H_2O_2 *in vivo*. We first cloned the YLR109 sequence in the inducible shuttle plasmid YCp2, and overexpressed the corresponding protein in EMY60 wild-type cells on YNBRGal medium. These cells were plated at various dilutions on YNBRGal medium containing several dilutions of H_2O_2, and growth on this medium was compared with wild-type cells (Fig. 6). As indicated by the result of the experiment, YLR109 protects efficiently against H_2O_2 *in vivo*, since the cells overexpressing YLR109 are able to grow at low cell concentration at 0.8 mM H_2O_2. Indeed, these cells overexpressing YLR109 are even able to grow on a medium containing up to 1.2 mM H_2O_2 (not shown), a concentration which is lethal for wild-type cells.

YLR109 and Its Arabidopsis Homologue AtTPX2 Present a Thioredoxin-dependent Peroxidase Activity in Vitro—The ability of HisAtTRX3C35S to reduce H_2O_2 tolerance in EMY60 (dominant negative phenotype), the ability of YLR109 to increase H_2O_2 tolerance in EMY60 and the similarity of YLR109 to thioredoxin peroxidases suggest that YLR109 and the other unidentified proteins of the same phylogenic group may be thioredoxin-dependent peroxidases. One characteristic of the peroxiredoxin family to which YLR109 belongs is the ability to form dimers. To test the property of YLR109 to form such a structure, recombinant His-tagged YLR109 protein was produced in *E. coli* and purified from the soluble fraction of the bacterial cells. One part of the purified protein was diluted in SDS sample buffer in the presence of β-mercaptoethanol and incubated at room temperature for 5 min (denaturing reducing condition). The second part was diluted in SDS sample buffer in the absence of β-mercaptoethanol (denaturing nonreducing condition). Both samples were analyzed by SDS-PAGE (Fig. 7). The His-tagged version of YLR109 is mainly present as a dimer of 42 kDa under nonreducing conditions, whereas it is present as a monomer of 23 kDa under reducing conditions. Both subunits are bridged by a disulfide bond, which can be reduced by β-mercaptoethanol, releasing 23-kDa monomers.

Other *in vitro* tests were also set up in order to characterize TPx activity further. We first used "the plasmid protection test:" in the presence of an electron donor such as DTT or ascorbate, Fe^{3+} catalyzes the reduction of O_2 to H_2O_2, which is further converted by the Fenton reaction to hydroxyl radicals ($HO^{\bullet}$) (32). These reactive oxygen species can inflict damage on various biomolecules, including proteins and DNA. In this test, TPx are known to prevent such damage by removing H_2O_2 preventing the Fenton reaction. We therefore investigated whether YLR109 can protect DNA from damage induced by this metal-catalyzed system (Fig. 8). In our plasmid protection test, 0.32 mM DTT in the presence of 3 μM $FeCl_3$ were able to degrade 1 μg of plasmid DNA. Both $FeCl_3$ and DTT are necessary for plasmid degradation as shown by the smear observed in Fig. 8. 20 μM YLR109 efficiently protects DNA while 20 μM bovine serum albumin is not efficient. This result is in agreement with a redox-dependent peroxidase activity of this protein.

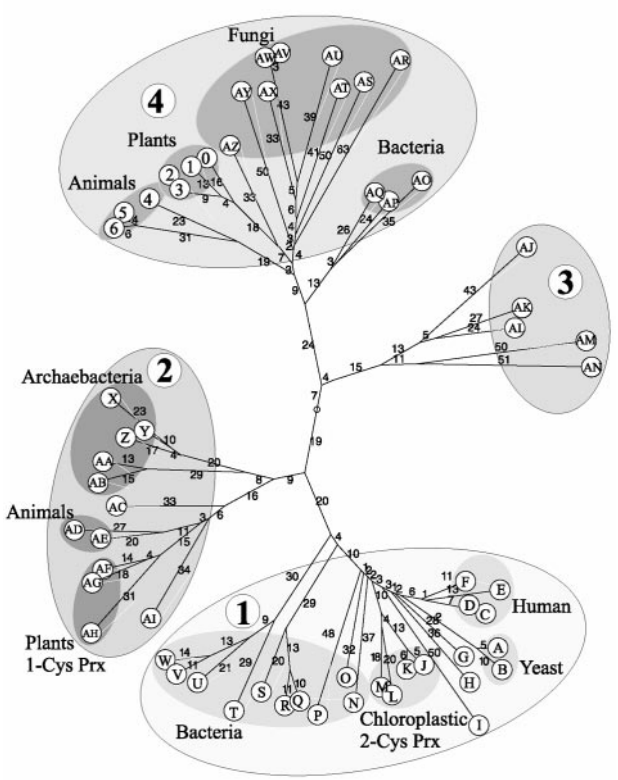


FIG. 5. **Phylogenetic tree of proteins homologous to thioredoxin dependent-peroxidase.** The DARWIN software (29) was used to generate this tree. EMBL or accession number is indicated for represented organisms. Cluster 1: 2-Cys thiol peroxidases. Yeast: *A,* P34760 YML028 *S. cerevisiae*; *B,* 927720 YDR453 *S. cerevisiae.* Human: *C,* L19185 HKEFA *Homo sapiens*; *D,* Q06830 PAG *H. sapiens*; *E,* U25182 AOE37 *H. sapiens*; *F,* X82321 TSAOX *H. sapiens*; *G,* U26666 *Trypanosoma brucei*; *H,* U88577 *Fasciola hepatica*; *I,* S67947 *Entamoeba histolytica.* Chloroplatic 2-Cys Prx: *J,* Q96291 *A. thaliana*; *K,* Z34917 BAS1 *Hordeum*; *L,* D64000 orf sl1010785 BAS1 *Synechocystis*; *M,* U38804 BAS1 *Porphyra.* Bacteria: *N,* M60116 *Salmonella typhimurium*; *O,* Z99111 *Bacillus subtilis*; *P,* AE000654 *Helicobacter pylori*; *Q,* U24084 *Mycobacterium bovis*; *R,* U31978 *Mycobacterium smegmatis*; *S,* U18620 *Corynebacterium diphtheria*; *T,* U94336 *Xanthomonas campestris*; *U,* A35441 *Salmonella typhimurium*; *V,* S52934 *Staphylococcus aureus*; *W,* P80239 *B. subtilis*. Cluster 2:, 1-Cys thiol peroxidases. Archaebacteria: *X,* AE000804 *Methanobacterium fulgidus*; *Y,* AE001087 *Archaeolus fulgidus*; *Z,* H64391 *Methanococcus jannaschii*; *AA,* U36479 *Sulfolobus*; *AB,* AF007757 *Sulfolobus metallicus*; *AC,* P34227 YLB064 *Saccharomyces cerevisiae.* Animals: *AD,* P30041 *H. sapiens*; *AE,* P52570 Rehydrin *Onchocerca.* Plants: *AF,* P52571 Rehydrin *Bromus*; *AG,* Y12089 Rehydrin *A. thaliana*; *AH,* U40818 Rehydrin *Tortula ruralis*; *AI,* D1018112 *Synechocystis*. Cluster 3, bacterioferritin comigrating proteins. *AJ,* AL021185 *Mycobacterium*; *AK,* U32711 *Hemophilus*; *AL,* M63654 *E. coli*; *AM,* U14189 *Plasmodium falci*; *AN,* P40553 YIL010 *S. cerevisiae*. Cluster 4, YLR109 and homologues. Bacteria, *AO,* U32739 *Heamophilus*; *AP,* D90909 *Synechocystis*; *AQ,* X72888 orf sll1621 *Rhizobium capsulatus.* Fungi, *AR,* AJ002536 *Schizosaccharomyces*; *AS,* U53878 YLR109 *S. cerevisiae*; *AT,* U11244 *Lipomyces*; *AU,* AB011805 *Malassezia furfur*; *AV,* P14293 PMP20b *C. boidini*; *AW,* P14292 PMP20a *C. boidini*; *AX,* AB011804 *M. furfur*; *AY,* U58050 *Aspergillus*; *AZ,* EST contig translation *Dictyostelium.* Plants**:** *0,* EST contig translation Rice; *1,* EST contig translation *Populus*; *2,* AtTPX2 *A. thaliana*; *3,* AtTPX1 *A. thaliana.* Animals: *4,* EST contig translation *Drosophila*; *5,* EST contig translation *H. sapiens*; *6*, EST contig translation mouse.

The thioredoxin dependence of the peroxidase activity of YLR109 was demonstrated by constructing a complete reduction system with recombinant proteins purified from *E. coli*: Trx reductase (NTR) was produced from an *A. thaliana* clone (33), Trx h from *C. reinhardtii* (34) or AtTRX3 from *A. thaliana* (5), YLR109 from *S. cerevisiae*. In this test Trx is reduced by the NADPH Trx reductase in the presence of the electron donor

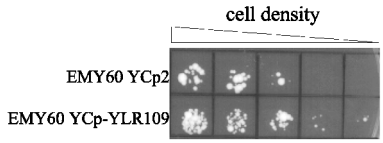


FIG. 6. **H_2O_2 tolerance of wild-type yeast EMY60 overexpressing or not YLR109.** Cells were first grown in YNBRGal medium to a density of about 10^7 cells per ml, and then diluted to $OD_{600\ nm}$ = 0.2. Dilutions from 5 to 5 were carried out and 15 μl of each were plated on YNBRGal medium containing 0.8 mM H_2O_2, and incubated 3 days at 30 °C.

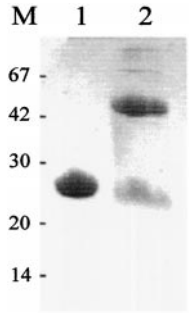


FIG. 7. **Effect of DTT on the dimerization of YLR109.** 10 μg of purified recombinant HisYLR109 were incubated for 5 min at room temperature under reducing conditions, with 5% β-mercaptoethanol (*lane 1*) or nonreducing conditions, without β-mercaptoethanol (*lane 2*), submitted to a 15% SDS-PAGE and stained by Coomassie Blue. Positions of molecular size standards are indicated in kilodaltons (*lane M*).

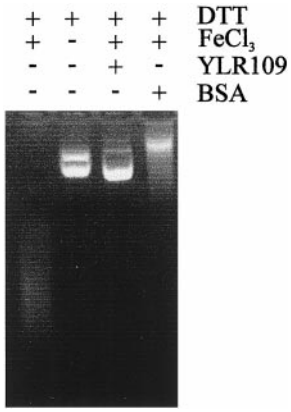


FIG. 8. **YLR109-dependent inactivation of DNA cleavage by a metal-catalyzed oxidation system.** Reactive oxygen species were generated for 30 min by addition of 0.32 mM DTT in the presence of 3 μM $FeCl_3$. Reactions mixtures were incubated at room temperature with 20 μM YLR109 and 1 μg of plasmid DNA for 4 h. The control tube contains 20 μM bovine serum albumin instead of YLR109. DNA was electrophoresed in a 1% agarose gel and stained by ethidium bromide.

NADPH. Trx can in turn be used as a substrate by the putative Prx to reduce H_2O_2. Thus there is a direct relationship between the oxidation of NADPH and the amount of H_2O_2 reduced. The putative Prx YLR109 was tested for its capacity to reduce H_2O_2, following the coupled NADPH oxidation (35). Table I shows the requirements of the H_2O_2 reduction assay. No NADPH oxidation was recorded in the absence of either NTR or thioredoxin indicating that these proteins were necessary for transmitting the reducing power. The reaction was also strictly dependent both on the presence of YLR109 and H_2O_2. Thus, under these conditions YLR109 is able to reduce H_2O_2 using electrons from NADPH (see also Fig. 9*A*). This clearly establishes that YLR109 is a Trx-dependent peroxidase. The H_2O_2 reducing activity was dependent on the amount of Prx added (Fig. 9*A*). Its specific activity of 560 μmol of NADPH oxidized min^{-1} per mg of $YLR109^{-1}$ and thus 560 μmol of H_2O_2 oxidized

TABLE I
Requirements for the Prx-dependent removal of H_2O_2

Assay	$\delta A_{340} \cdot min^{-1}$
Complete, 10 μg of YLR109	0.035
Complete, 20 μg of YLR109	0.07
Complete, 50 μg of YLR109	0.16
Minus YLR109	0
Minus Trx	0
Minus NTR	0
Minus H_2O_2	0

min^{-1} per mg of $YLR109^{-1}$ does not differ significantly from values reported previously for classical 2-Cys TPx (35, 36). In this assay system, the polyhistidine tail of YLR109 does not seem to not prevent this protein from being an efficient catalyst for H_2O_2 reduction.

The effect of increasing H_2O_2 concentrations on the Prx activity was tested. The reaction exhibited a Michaelis type saturation with a K_m H_2O_2 of about 14 μM (Fig. 9*B*). In addition, H_2O_2 inactivates the Prx especially at high concentration (36). This is apparent in the kinetics of NADPH oxidation at 340 nm, the rate of which decreases as time increases (data not shown).

The YLR109 protein used *C. reinhardtii* Trx h as a substrate with a very good affinity (K_m about 1 μM). This type of Trx possesses a canonical active site (WCGPC). We have also tested in the same conditions AtTRX3 which possesses a WCPPC active site. The kinetics of saturation with this alternate donor were extremely similar to those obtained with the WCGPC type with a K_m nearly identical (data not shown).

We have produced and purified a His-tagged version of the protein encoded by clone 149H22T7, which encodes AtTPx2 from *Arabidopsis*. In the same *in vitro* test, AtTPx2 shows a typical Trx-dependent peroxidase activity, but the protein is far less stable that its *Saccharomyces* counterpart.

DISCUSSION

Possible Strategies to Characterize Proteins Interacting in Vivo with Thioredoxins and Glutaredoxins in the Thiol-mediated Redox Cascade—In this report, we describe an improved biochemical system for purifying the target proteins of Trx implicated in thiol reduction. Most disulfide-regulated proteins can be activated *in vitro* by Trx in an almost unspecific way. On the other hand, genetic evidence suggests that Trx undergo specific interactions with a limited number of proteins *in vivo*. In order to identify unambiguously the function of Trx, characterization of *in vivo* thioredoxin-protein complexes is needed. Presently the most popular method to characterize protein-protein interactions is the two-hybrid system which was very efficient in defining kinase/phosphatase cascades. Up to now, only three articles report on two-hybrid characterization of Trx complexes (14, 15, 37). But in these cases, it is not clear whether Trx participates in a redox cascade. In our group, we were unable to isolate putative AtTRX3 targets from an *Arabidopsis* two-hybrid library. Immunoprecipitations using anti-TRX antibodies were no more efficient. The most probable cause is that the half-life of the TRX-target complexes is very short. The second difficulty is the relatively low abundance of Trx *in vivo*.

The biochemical approach that we have developed in this study solves both aspects. The mutation of the second cysteine in the catalytic site of the Trx stabilizes the mixed disulfide intermediate which can be efficiently isolated by Ni^{2+} chromatography, involving the N-terminal polyhistidine extension added to the Trx. The isolation of YLR109 shows that at least this mixed disulfide intermediate is sufficiently stable *in vivo* to allow the isolation of the complex. Surprisingly, this complex could be isolated only in the Δ Trx yeast presumably because the wild-type Trx attack the disulfide bridge of the mixed disulfide. This suggests that the two-hybrid approach could be efficiently improved using a C35S Trx mutant as bait and, if that was not sufficient, by using a Δ Trx yeast as reporter strain. We have recently screened a yeast two-hybrid library with a C35S mutant of YTRX1, one of the yeast Trx, fused to the GAL4-binding domain and isolated several putative Trx targets which are now under study. Nevertheless YLR109 was not among these clones. More surprisingly, a binary two-hybrid system with YTRX1C35S fused to the activation domain of GAL4 and YLR109 fused to the DNA-binding domain of GAL4 failed to show interaction. We have no interpretation of this result, but the large amount of free YLR109 in yeast probably competes with the hybrid GAL4-YLR109 protein in the interaction with the hybrid GAL4-Trx. Despite our success in isolating YLR109, no other protein target has yet been isolated by this method, although AtTRX3 is not only able to confer H_2O_2 tolerance but also induces a normal cell cycle and rapid growth on methionine sulfoxide as sole sulfur source. The present failure to detect additional complexes may be due to their low concentration, or possibly to an interaction that limits the efficiency of the retention on the Ni^{2+} affinity column. A lower stability of these mixed disulfides in the Δ Trx yeast cannot be excluded because it remains able to synthesize other reducers, like glutathione and Grx. Our results clearly show that both methods are complementary and suggest modifications of the two-hybrid approach that could be necessary to detect low abundance targets. In addition, the recent characterization of stable mixed disulfides between the *E. coli* Grx1 (C14S) and a peptide from the ribonucleotide reductase B1 suggests that the same methods could be used for the characterization of Grx targets (38). Furthermore, mixed disulfide intermediates have been obtained from *E. coli* with glutathione, by mutating the Grx in the more N-terminal cysteine of it active site C14S (39, 40), and also between *E. coli* TrxA C32S and its Trx reductase (41). Thus, a similar approach would be likely to help to discriminate between the possible reducers of Trx and Grx in the case of multiple thioredoxin reductase genes, as is the case for *A. thaliana*, or to identify the reducer when this one is not known, as for chloroplastic APS reductase from *A. thaliana*. This protein shows homology to Trx but is reduced by glutathione *in vitro* and in *E. coli* (6).

YLR109 Defines a New Group of Peroxiredoxins—Our biochemical method to identify Trx targets led us to isolate and to characterize a new target in yeast (YLR109), of unknown function up to now. In contrast to most peroxidases which use cofactors to reduce H_2O_2, YLR109 and its related proteins belong to the recently characterized family of Prx, a set of enzymes which transfer their reducing power by means of a cysteine.

The first member which defines the first group of this large family was discovered in yeast and first named thiol-specific antioxidant (30), but characterized later as a true peroxidase and renamed 2-Cys Prx. This protein catalyzes the reduction of H_2O_2 and alkyl hydroperoxides *in vitro* with the use of electrons from the Trx system (35). Yeast Prx exists as a homodimer and contains two essential Cys residues in each subunit. The Cys^{47}-SH group is the primary site of oxidation by H_2O_2, and the oxidized Cys (probably a sulfenic acid form, Cys-SOH) rapidly reacts with the Cys^{170}-SH of the other subunit to form an intermolecular disulfide. This disulfide is subsequently reduced by a Trx, and mutant TPx proteins lacking either Cys^{47} or Cys^{170} therefore do not exhibit Trx-coupled peroxidase activity (42). 2-Cys Prx corresponds to cluster 1 of the tree on Fig. 5. *S. cerevisiae* presents two very similar 2-Cys

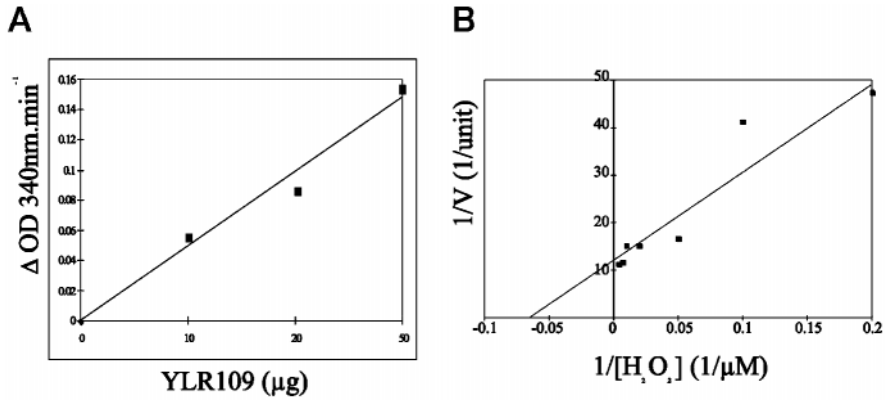


FIG. 9. **Thioredoxin-dependent reduction of hydrogen peroxide.** *A,* effect of various concentrations of YLR109 (0.5–2.5 μM) on NADPH removal in a 1 ml of reaction medium containing 30 mM Tris-HCl, pH 8.0, 0.2 mM NADPH, 0.18 μM *A. thaliana* NTR, 0.67 μM *C. reinhardtii* Trx h, and 100 μM H_2O_2. The reaction was initiated by the addition of either NTR or H_2O_2. *B,* effect of various concentrations of H_2O_2 (1–200 μM) on NADPH removal in a 1-ml reaction medium containing 30 mM Tris-HCl, pH 8.0, 0.2 mM NADPH, 0.18 μM *A. thaliana* NTR, 0.67 μM *C. reinhardtii* Trx h, and 100 μM YLR109. The reaction was initiated by the addition of NTR.

Prx genes, humans have at least four different genes. In higher plants, all 2-Cys Prx described so far are nuclear-encoded chloroplastic proteins.

The second group of Prx possesses only one conserved cysteine residue and is consequently designated as 1-Cys Prx. The first member was characterized as an antioxidant in barley seeds (43), then homologues were found in most plants, archebacteria, and animals (cluster 2 in our phylogenetic analysis on Fig. 5). The human 1-Cys does not form a disulfide and DTT acts *in vitro* as an efficient reducer of the 1-Cys Prx, but the natural electron donor remains unidentified, glutathione and Trx being inefficient (44). Recent advances on crystal structure of this human Prx reveals that the C-terminal domain of this protein is used for dimerization, and that the active site cysteine (Cys^{47}) exists as cysteine-sulfenic acid in the crystal (45).

In this work, we have demonstrated the peroxidase activity for YLR109 and AtTPX2, two distant members of the group. This suggests that all the members that we have identified in our phylogenic analysis (group 4 on Fig. 5) should be Prx. Our work shows that the NADPH/Trx reductase/Trx system is a very efficient electron donor for these Prx *in vitro*. Thus, despite the slightly higher sequence similarity of YLR109 homologues with 1-Cys Prx and the presence of only one conserved Cys (Cys^{62} for YLR109), these proteins seem to be functionally closer to 2-Cys TPx than to 1-Cys TPx. This is supported by our experiment showing that YLR109, like TPx, can adopt a disulfide-bonded dimeric structure (Fig. 6A). Furthermore, we show that YLR109 is dependent on a functional NADPH/Trx reductase/Trx system to reduce H_2O_2. Finally, our study is the first evidence for an *in vivo* interaction between a Prx and the Trx system.

Physiological Function of YLR109 and the Thioredoxin Reduction System—We have previously shown that *AtTRX3* can restore H_2O_2 tolerance to the budding yeast EMY63, lacking the two Trx genes (13). This means that AtTRX3 interacts specifically with a protein involved in H_2O_2 tolerance. Our results are in good agreement with recent two-dimensional analysis, since after H_2O_2 treatment of *S. cerevisiae* cells, the amount of Trx and YLR109 increases 11 and 3 times, respectively (28). These authors suggest that YLR109 may be an antioxidant protein. We demonstrate that AtTRX3C35S interacts strongly with YLR109 and, at the same time, induces a partial dominant negative phenotype essentially limited to H_2O_2 tolerance. In addition, YLR109 presents a Trx-dependent peroxidase activity *in vitro* and overexpression of this ORF in EMY60 increases H_2O_2 tolerance. All these facts suggest that *in vivo,* Trx transfers reducing equivalents from NADPH to YLR109 through a thiol-mediated cascade allowing the degradation of hydrogen peroxide. Thus, in this interaction, the NADPH/Trx reductase/AtTRX3 cascade appears to transfer an energetic flux rather than to modify the structure of the targeted protein.

Nevertheless, the external application of H_2O_2 is an artificial situation which is probably experienced by yeast and other organisms only in the laboratory. This poses the question of the real function of YLR109 and its Trx-mediated reduction. YLR109 and AtTPX2 are abundant cytosolic proteins as shown on the proteomes, and the number of ESTs in plants and mammals indicates that the corresponding genes are also very actively transcribed in these organisms. The simplest hypothesis is that YLR109 homologues eliminate the excess H_2O_2 or other peroxides, like alkyl hydroperoxides, produced by metabolism. In relation to this hypothesis it is important to remember that PMP20a and PMP20b, two YLR109 homologues from *C. boidini*, are peroxisomal proteins. Furthermore, the conservation of the C-terminal end of all eukaryotic YLR109 homologues suggests a peroxisomal location. Peroxisomes are a major source of H_2O_2 production due to fatty acid degradation in all eukaryotes and to photorespiration in plants. Thus, these Prx may help catalase in the elimination of H_2O_2 from peroxisomes, to prevent its diffusion into the cytosol, and/or may reduce membrane bound alkyl-hydroperoxides, for which catalases are inefficient. Control of cytoplasmic H_2O_2 concentration is crucial for cells, since diverse stimuli have been shown to use reactive oxygen species (*e.g.* H_2O_2) as transduction signals for regulating transcription factors like NF-κB, AP1, and OxyR, via the formation of an internal disulfide bridge (46–48). In mammalian cells, the tumor necrosis factor α and growth factors (epidermal growth factor and platelet-derived growth factor) are known to induce a transient increase in intracellular concentration of H_2O_2 (49). It was recently shown that the overproduction of the mammalian Prx II blocks the NF-κB activation induced by exogenous H_2O_2 or tumor necrosis factor α (44). Moreover, the activation of NF-κB was also prevented by a rapid removal of H_2O_2 by catalases (50). These data reinforce a possible function of Prx in H_2O_2 removal. Human 2-Cys Prx (TPx II) was also characterized as a potent inhibitor of cytochrome *c* release from mitochondria to cytosol, and of lipid peroxidation in cells (51). In all these cases, this TPx II could protect cells from apoptosis. In higher plants, H_2O_2 is a well established signal in response to wounding (52) and pathogen interactions (53–55). Furthermore, recent evidence shows that sulfhydryl blockers induce an H_2O_2 burst (56). Thus, Trx-dependent peroxidases could play a central role in signal

transduction and in response to pathogens. Isolation of YLR109 mutants in yeast and of the homologues in other organisms will probably be necessary to define the implication of these proteins and their Trx-reduction dependence in a general antioxidant mechanism, and/or in a more subtle function in signaling pathways.

Acknowledgments—We thank Richard Cooke (Perpignan) for reading the manuscript and Patrick Monfort (UMR CNRS 5556, Montpellier) for flow cytometry analyses.

Note Added in Proof—Since this paper was submitted, two other articles describing YLR109 peroxidase function have been published (58, 59).

REFERENCES

1. Holmgren, A. (1985) *Annu. Rev. Biochem.* **54,** 237–271
2. Jacquot, J. P., Lancelin, J. M., and Meyer, Y. (1997) *New Phytol.* **136,** 543–570
3. Buchanan, B. B., Schürmann, P., and Jacquot, J. P. (1994) *Semin. Cell Biol.* **5,** 285–293
4. Schenck, H., Klein, M., Erdbrügger, W., and Dröge, W. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1672–1676
5. Rivera-Madrid, R., Mestres, D., Marinho, P., Jacquot, J. P., Decottignies, P., Miginiac-Maslow, M., and Meyer, Y. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5620–5624
6. Gutierrez-Marcos, J. F., Roberts, M. A., Campbell, E. I., and Wray, J. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13377–13382
7. Laughner, B. J., Sehnke, P. C., and Ferl, R. J. (1998) *Plant Physiol.* **118,** 987–996
8. Li, X., Nield, J., Hayman, D., and Langridge, P. (1995) *Plant J.* **8,** 133–138
9. Rey, P., Pruvot, G., Becuwe, N., Eymery, F., Rumeau, D., and Peltier, G. (1998) *Plant J.* **13,** 97–107
10. Setya, A., Murillo, M., and Leustek, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13383–13388
11. Muller, E. G. (1991) *J. Biol. Chem.* **266,** 9194–9200
12. Muller, E. G. (1996) *Mol. Biol. Cell* **7,** 1805–1813
13. Mouaheb, N., Thomas, D., Verdoucq, L., Monfort, P., and Meyer, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3312–3317
14. Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3633–3638
15. Bower, M. S., Matias, D. M., Fernandes-Carvalho, E., Mazzurco, M., Gu, T., Rothstein, S. J., and Goring, D. R. (1996) *Plant Cell* **8,** 1641–1650
16. Kallis, G. B., and Holmgren, A. (1980) *J. Biol. Chem.* **255,** 10261–10265
17. Wynn, R., Cocco, M. J., and Richards, F. M. (1995) *Biochemistry* **34,** 11807–11813
18. Qin, J., Clore, G. M., Poindexter Kennedy, W. M., Huth, J. R., and Gronenborn, A. M. (1995) *Structure* **3,** 289–297
19. Cole, G. M., Stone, D. E., and Reed, S. I. (1990) *Mol. Cell. Biol.* **10,** 510–517
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Ford, N., Nolan, C., and Ferguson, M., eds) pp. 1.7–1.85, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Shermann, F. (1991) in *Guide to Yeast Genetics and Molecular Biology* (Guthrie, C., and Fink, G. R., eds) pp. 3–21, Academic Press, San Diego, CA
22. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
23. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1988) *Gene* (*Amst.*) **77,** 51–59
24. Holmgren, A. (1979) *J. Biol. Chem.* **254,** 9627–9632
25. Klimowski, L., Chandrashekar, R., and Tripp, C. A. (1997) *Mol. Biochem. Parasitol.* **90,** 297–306
26. Arnold, F. H. (1991) *Bio/Technology* **9,** 151–156
27. Garrard, L. J., and Goodman, J. M. (1989) *J. Biol. Chem.* **264,** 13929–13937
28. Godon, C., Lagniel, G., Lee, J., Buhler, J. M., Kieffer, S., Perrot, M., Boucherie, H., Tolenado, M. B., and Labarre, J. (1998) *J. Biol. Chem.* **273,** 22480–22489
29. Gonnet, G. H., Cohen, M. A., and Benner, S. A. (1992) *Science* **256,** 1443–1445
30. Kim, K., Kim, I. H., Lee, K-Y., Rhee, S. G., and Stadtman, E. R. (1988) *J. Biol. Chem.* **263,** 4704–4711
31. Nagase, T., Miyajima, N., Tanaka, A., Sazuka, T., Seki, N., Sato, S., Tabata, S., Ishikawa, K., Kawarabayasi, Y., Kotani, H., and Nomura, N. (1995) *DNA Res.* **2,** 37–43
32. Gutteridge, J. M., and Halliwell, B. (1989) *Bailliere's Clin. Haematol.* **2,** 195–256
33. Jacquot, J. P., Rivera-Madrid, R., Marinho, P., Kollarova, M., Le Marechal, P., Miginiac-Maslow, M., and Meyer, Y. (1994) *J. Mol. Biol.* **235,** 1357–1363
34. Stein, M., Jacquot, J. P., Jeannette, E., Decottignies, P., Hodges, M., Lancelin, J. M., Mittard, V., Schmitter, J. M., and Miginiac-Maslow, M. (1995) *Plant Mol. Biol.* **28,** 487–503
35. Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) *J. Biol. Chem.* **269,** 27670–27678
36. Cha, M. K., Kim, H. K., and Kim, I. K. (1995) *J. Biol. Chem.* **270,** 28635–28641
37. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) *EMBO J.* **17,** 2596–2606
38. Berardi, M. J., Pendred, C. L., and Bushweller, J. H. (1998) *Biochemistry* **37,** 5849–5857
39. Bushweller, J. H., Aslund, F., Wuthrich, K., and Holmgren, A. (1992) *Biochemistry* **31,** 9288–9293
40. Bushweller, J. H., Billeter, M., Holmgren, A., and Wuthrich, K. (1994) *J. Mol. Biol.* **235,** 1585–1597
41. Wang, P. F., Veine, D. M., Ahn, S. H., and Williams, C. H., Jr. (1996) *Biochemistry* **35,** 4812–4819
42. Chae, H. Z., Uhm, T. B., and Rhee, S. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3633–3638
43. Stacy, R. A., Munthe, E., Steinum, T., Sharma, B., and Aalen, R. B. (1996) *Plant Mol. Biol.* **31,** 1205–1216
44. Kang, S. W., Chae, H. Z., Seo, M. S., Kim, K., Baines, I. C., and Rhee, S. G. (1998) *J. Biol. Chem.* **273,** 6297–6302
45. Choi, H. J., Kang, S. W., Yang, C. H., Rhee, S. G., and Ryu, S. E. (1998) *Nat. Struct. Biol.* **5,** 400–406
46. Abate, C., Patel, L., Rauscher, F. J., and Curran, T. (1990) *Science* **249,** 1157–1161
47. Meyer, M., Schreck, R., and Baeuerle, P. A. (1993) *EMBO J.* **12,** 2005–2015
48. Zheng, M., Aslund, F., and Storz, G. (1998) *Science* **279,** 1718–1721
49. Meier, B., Radeke, H. H., Selle, S., Younes, M., Sies, H., Resch, K., and Habernehl, G. G. (1989) *Biochem. J.* **263,** 539–545
50. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 2247–2258
51. Zhang, P., Liu, B., Kang, S. W, Seo, M. S., Rhee, S. G., and Obeid, L. M. (1997) *J. Biol. Chem.* **272,** 30615–30618
52. Rea, G., Laurenzi, M., Tranquilli, E., D'Ovidio, R., Federico, R., and Angelini, R. (1998) *FEBS Lett.* **437,** 177–182
53. Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A., and Lamb, C. (1998) *Cell* **92,** 773–784
54. Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H., Jr., Van Montagu, M., Inze, D., and Van Camp, W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5818–5823
55. Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van Montagu, M., Inze, D., and Van Camp, W. (1997) *EMBO J.* **16,** 4806–4816
56. Marre, M. T., Amicucci, E., Zingarelli, L., Albergoni, F., and Marre, E. (1998) *Plant. Physiol.* **118,** 1379–1387
57. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
58. Jeong, J. S., Kwon, S. J., Kang, S. W., Rhee, S. G., and Kim, K. (1999) *Biochemistry* **38,** 776–783
59. Lee, J., Spector, D., Godon, C., Labarre, J., and Toledano, M. B. (1999) *J. Biol. Chem.* **274,** 4537–4544