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The Role and Regulation of Trx1, a Cytosolic Thioredoxin in *Schizosaccharomyces pombe*

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The genome of fission yeast *Schizosaccharomyces pombe* harbors two genes for thioredoxins, *trx1*$^+$ and *trx2*$^+$, which encode cytosolic and mitochondrial thioredoxins, respectively. The Δ*trx1* mutant was found sensitive to diverse external stressors such as various oxidants, heat, and salt, whereas Δ*trx2* mutant was not sensitive except to paraquat, a superoxide generator. Both Δ*trx1* and Δ*trx2* mutants were more resistant to diamide, a thiol-specific oxidant, than the wild type. The *trx1*$^+$ gene expression was induced by H_2O_2 and menadione, being mediated through a stress-responsive transcription factor Pap1. In Δ*trx1* cells, the basal expression of Pap1-regulated genes were elevated, suggesting a role for Trx1 as a reducer for oxidized (activated) Pap1. The Δ*trx1* mutant exhibited cysteine auxotrophy, which can be overcome by adding sulfite. This suggests that Trx1 serves as a primary electron donor for 3'-phosphoadenosine-5'-phosphosulfate (PAPS) reductase and thus is an essential protein for sulfur assimilation in *S. pombe*. These results suggest that, in contrast to Trx2 whose role is more confined to mitochondrial functions, Trx1 plays a major role in protecting *S. pombe* against various stressful conditions and enables proper sulfur metabolism.

***Keywords*:** fission yeast, oxidative stress, thioredoxin, mitochondria, Pap1, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase

All aerobic organisms which are exposed to reactive oxygen species (ROS) are equipped with systems that reduce oxidized cell components. The reducing system includes thiol-specific molecules such as glutathione (GSH), thioredoxin (Trx) and glutaredoxin (Grx) systems. They contain redox-active dithiol or monothiol cysteines and contribute to redox homeostasis of the cell by reducing disulfide bonds (Carmel-Harel and Storz, 2000; Herrero and Ros, 2002; Masutani and Yodoi, 2002; Vlamis-Garadikas and Holmgren, 2002). As thiol-buffer molecules, GSH, Grx, and Trx share some overlapping functions. However, in each organism, the specific role and contribution could be different. In *Escherichia coli*, either Grx or Trx system can contribute to maintain highly reduced GSH in the absence of glutathione reductase (GR) (Tuggle and Fuchs 1985; Russel and Holmgren, 1988), and even GSH-negative cells are as resistant as wild type against oxidative stress (Greenberg and Demple, 1986). In yeast *Saccharomyces cerevisiae*, which contain two cytosolic (TRX1, TRX2) and one mitochondrial (TRX3) thioredoxins (Pedrajas *et al*., 1999), the cytosolic Trx system is thought to provide an overlapping function with GR, since Δ*trx1*Δ*trx2* double mutant accumulates oxidized GSSG as in Δ*glr1* mutant deficient in GR, and the Δ*glr1*Δ*trx1*Δ*trx2* triple mutant is not viable (Muller, 1996).

Thioredoxins (Trxs) function as antioxidants that efficiently reduce disulfide bonds in a variety of substrates and provide electrons to thioredoxin dependent peroxidases due to its low redox potential (-270 mV) (Holmgren, 1984). It is re-reduced by thioredoxin reductase using NADPH (Masutani and Yodoi, 2002; Vlamis-Garadikas and Holmgren, 2002). Trxs also serve as electron donors for several enzymes such as ribonucleotide reductase for DNA synthesis, methionine sulfoxide reductase, and 3'-phosphoadenosyl-5'-phosphosulfate (PAPS) reductase for sulfur assimilation (Muller, 1991; Masutani and Yodoi, 2002). Trx can also modulate eukaryotic signal transduction pathway, as observed in NF-kB activation (Hirota *et al*., 1999; Okamoto *et al*., 2002) and in anti-apoptotic regulation (Saitoh *et al*., 1998). In *S. cerevisiae*, Yap1, a peroxide-sensitive transcriptional regulator of AP-1 family, is activated through disulfide bond formation that permits nuclear localization, and is reduced by Trx (Izawa *et al*., 1999; Delaunay *et al*., 2002).

In fission yeast *Schizosaccharomyces pombe*, the genome sequence reveals two genes for thioredoxin, *trx1*$^+$ and *trx2*$^+$. We previously observed that the overproduction of mitochondrial Trx2 can compensate the growth defect caused by depletion of reduced glutathione in *S. pombe*, and proposed that GSH is critically required for a mitochondrial function, such as to maintain Fe-S cluster from oxidation (Lee *et al*., 1997; Song *et al*., 2006). The role and regulation of cytosolic Trx1, in comparison with Trx2, has not been examined. In this study, we present evidence that Trx1 serves as a primary anti-oxidative system in *S. pombe* whose absence causes sensitivity toward oxidants and a defect in sulfur assimilation resulting in cysteine auxotrophy. The *trx1*$^+$ gene is induced by various oxidants, and its induction de-

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pends primarily on transcription factor Pap1.

Materials and Methods

Yeast strains and culture media

S. pombe strains used in this study are ED665 (*h⁻ ade6-M210 leu1-32 ura4-D18*), ED668 (*h⁺ ade6-M216 leu1-32 ura4-D18*), JY21d (*h⁻ ade6-M216 leu1-32 ura4-D18 trx1::ura4⁺*), and JY31b (*h⁺ ade6-M216 leu1-32 ura4-D18 trx2::ura4⁺*). Growth and maintenance of all the strains were generally done as described previously (Moreno *et al*., 1991; Alfa *et al*., 1993). The Δ*pap1* (TP108-3c) and Δ*atf1* (KS1497) cells were obtained from Drs. T. Toda and P. Russell. Cells were grown in YES (0.5% yeast extract and 3% dextrose) media or Edinburgh Minimal Medium (EMM) with appropriate supplements as described by Alfa *et al*. (1993).

Treatment with various stresses

Sensitivity to various external stresses was determined by spotting on YES plates containing the indicated reagents. Cells at early exponential-growth phase (OD_{595}~0.5) were spotted on plates, and incubated for 2~3 days at 30°C. Oxidizing agents used were menadione (vitamin K_3, non-salt form; ICN), paraquat (methyl viologen; Sigma), hydrogen peroxide (Fluka), cumene hydroperoxide (Sigma), and diamide (Sigma). Heat shock was applied by incubating plates at 37°C.

Construction of *trx* mutants

The *trx1⁺* disruptant was made by transforming ED665/ED668 diploid cell with 4.0 kb *Xba*I/*Eco*RI fragment that contain 1.8 kb *ura4⁺* gene cassette at the *Hin*dIII site in the *trx1* ORF and allowing homologous recombination at the *trx1⁺* locus. For disruption of *trx2⁺* gene, the *Bgl*II fragment in the *trx2* ORF was replaced with the *ura4⁺* gene cassette. The 3.2 kb *Hin*dIII fragment containing the recombinant construct was introduced to diploid cells. The transformants were selected by *ura+* marker and the correct disruption of the genes was confirmed by both colony PCR and genomic Southern hybridization. Following tetrad spore formation, the haploid Δ*trx1* strain was selected.

RNA preparation and Northern hybridization

Total RNA was prepared as described by Schmitt *et al*. (1990). Samples with 20 μg of total RNA were separated on agarose gel containing formaldehyde, transferred onto a Hybond-N^+ membrane (Amersham), and then fixed by UV-crosslinker (UVP). Hybridization was performed with the PCR product as a probe in Rapid-hyb buffer (Amersham) as recommended by the manufacturer. The signal was visualized by exposing the membrane to X-ray film and the radioactivity was quantified with PhosphoImager and Multi Gauge (Fuji).

Results and Discussion

S. pombe possesses two thioredoxins

There are two genes (*trx1⁺* and *trx2⁺*) for thioredoxin in the genome of *S. pombe* (Song *et al*., 2006; www.genedb.org/genedb/pombe). The encoded proteins exhibit significant similarity with other Trxs and contain the conserved Trp-Cys-Gly-Pro-Cys motif in the active site. Trx1 consists of 103 amino acids, sharing 51% identity and 74% similarity with *TRX2* in *S. cerevisiae*. In contrast to Trx2 which contain an obvious mitochondrial targeting sequence and shown to localize in mitochondria (Song *et al*., 2006), Trx1 does not contain any predictable signal sequence, and predicted to be localized in the cytosol. Both Trx1 and Trx2 proteins contain the disulfide reducing activity when provided with NADPH and Trr1, a thioredoxin reductase from *S. pombe* (Song, 2006). A third thioredoxin-like protein has been predicted in the genome (Trx3), and a recent study demonstrated that its expression is induced by oxidative stress in a Pap1-dependent manner. However, its function as a thioredoxin has not been demonstrated (Kim *et al*., 2007).

Role of thioredoxins in protection against external stresses

The Δ*trx1* and Δ*trx2* mutants grow as well as the wild type in rich YES media. We estimated their sensitivity toward various oxidants. Exponentially growing cells of the wild type, Δ*trx1*, and Δ*trx2* mutants were inoculated on plates containing various concentrations of hydrogen peroxide (H_2O_2), cumene hydroperoxide (CHP), menadione (MD, a superoxide generator), paraquat (PQ, a superoxide generator), and diamide (DA, a thiol-specific oxidant). The Δ*trx1* cells were more sensitive than the wild type to all the oxidants except diamide (Fig. 1A). It became sensitive to salt (0.2 M NaCl) and to heat (37°C). On the contrary, the Δ*trx2* cells did not show any significant difference in sensitivity toward hydrogen peroxide, cumene hydroperoxide, and menadione (Fig. 1B). It became sensitive only to paraquat. Both Δ*trx1* and Δ*trx2* mutants became resistant to diamide, suggesting that the thiol-oxidation of either thioredoxin by diamide could be harmful to cells, and the absence of thioredoxin could alleviate diamide toxicity. It can also be postulated that the lack of Trx may induce stress-responsive genes that protect cells from DA stress. These results demonstrate that Trx1 plays more crucial role than mitochondrial Trx2 in defending cells against oxidants. Trx1 may play a more general role as a protecting component toward broad range of stressors, not limited to oxidants.

Induction of thioredoxin genes by oxidative stress

We examined the effect of various oxidative stresses on the expression of *trx1⁺* and *trx2⁺* genes. When the exponentially growing wild type cells (OD_{595}~0.5) were treated with 1 mM H_2O_2, the *trx1⁺* mRNA increased by about 4-fold (Fig. 2A). Menadione at 50 μM also induced *trx1⁺* gene by more than 2-fold. Cumene hydroperoxide was slightly effective, whereas diamide and paraquat were not so effective in inducing *trx1⁺* gene. Even though menadione and paraquat are redox-cycling agents known to generate superoxides, it appears that menadione, a quinone compound, is a more effective superoxide generator in *S. pombe* (Lee *et al*., 1995) than a bipyridinium compound paraquat at concentrations examined. In order to evaluate whether transcriptional regulators that respond to oxidative stress are involved in this induction, we examined *trx1⁺* mRNA in the wild type and the mutants that lack Pap1 or Atf1. It was previously re-

ported that Pap1 controls the induction of target genes at lower concentration of H_2O_2 (0.1∼0.2 mM) and Atf1 takes part at higher concentration of H_2O_2 (>1 mM) (Quinn *et al*., 2002). As shown in Fig. 2B, the *trx1*$^+$ mRNA increased by 0.2 mM H_2O_2 in the wild type cell but not in Δ*pap1* mutant. In the Δ*atf1* mutant, the induction fold did not change. When the expression of *trx1*$^+$ gene was investigated in 1 mM H_2O_2- treated Δ*pap1* and Δ*atf1* strain, the result was not much different from that of the wild type (Fig. 2C). These results imply that Pap1, which is known to respond to low concentrations of H_2O_2, regulates the induction of *trx1*$^+$ gene. Compared with the *trx1*$^+$ gene, the *trx2*$^+$ gene

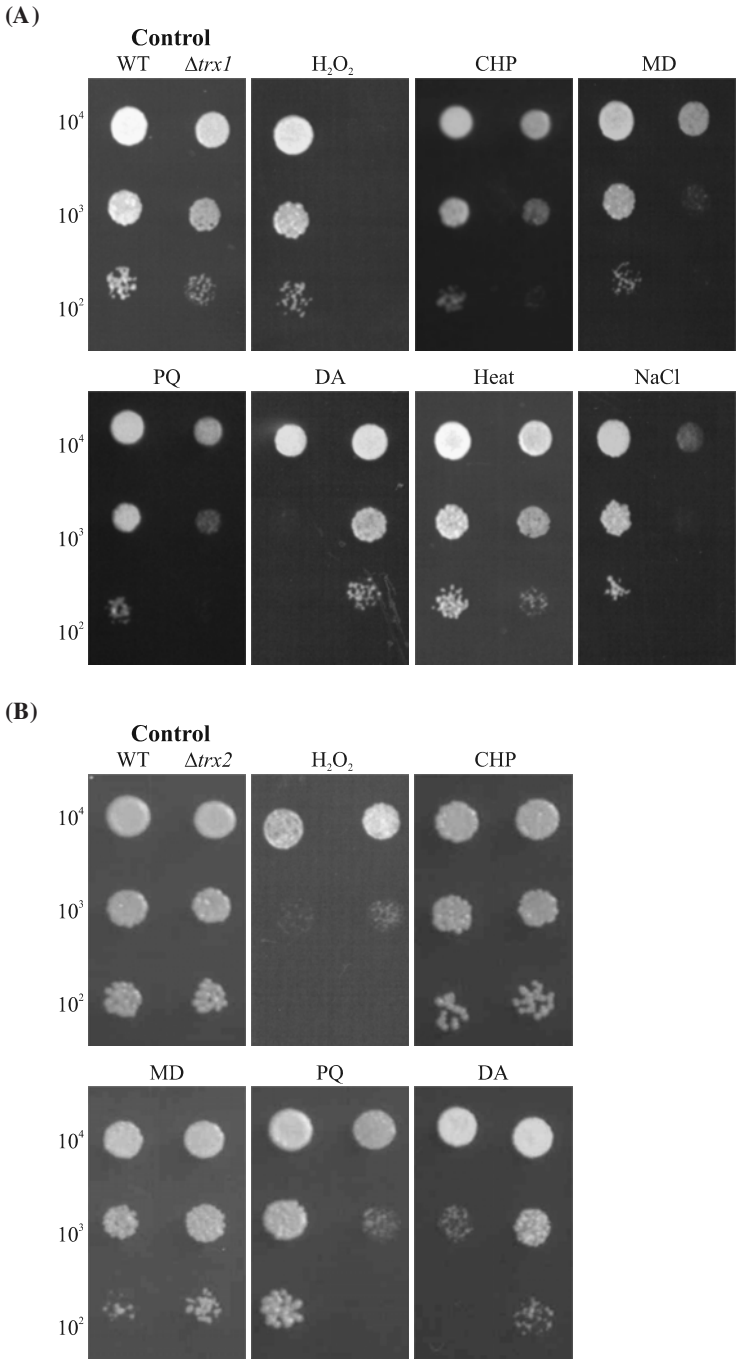

Fig. 1. Sensitivity of *trx* mutants to various external stresses. (A) Exponentially growing wild type (ED665) and Δ*trx1* (JY21d) cells were spotted on freshly prepared YES plates containing various kinds of reagents; 1 mM H_2O_2; 50 μM cumene hydroperoxide (CHP); 20 μM menadione (MD); 1 mM paraquat (PQ), 4 mM diamide (DA), or 0.2 M NaCl. The plates were incubated at 30°C for 2∼3 days, except for heat stress (37°C). The number of cells spotted on the plates is indicated on the left. (B) Exponentially growing wild type (ED668) and Δ*trx2* (JY31b) cells were spotted as in (A) on YES plates containing 2 mM H_2O_2; 100 μM CHP, 40 μM MD, 1 mM PQ; or 5 mM DA.

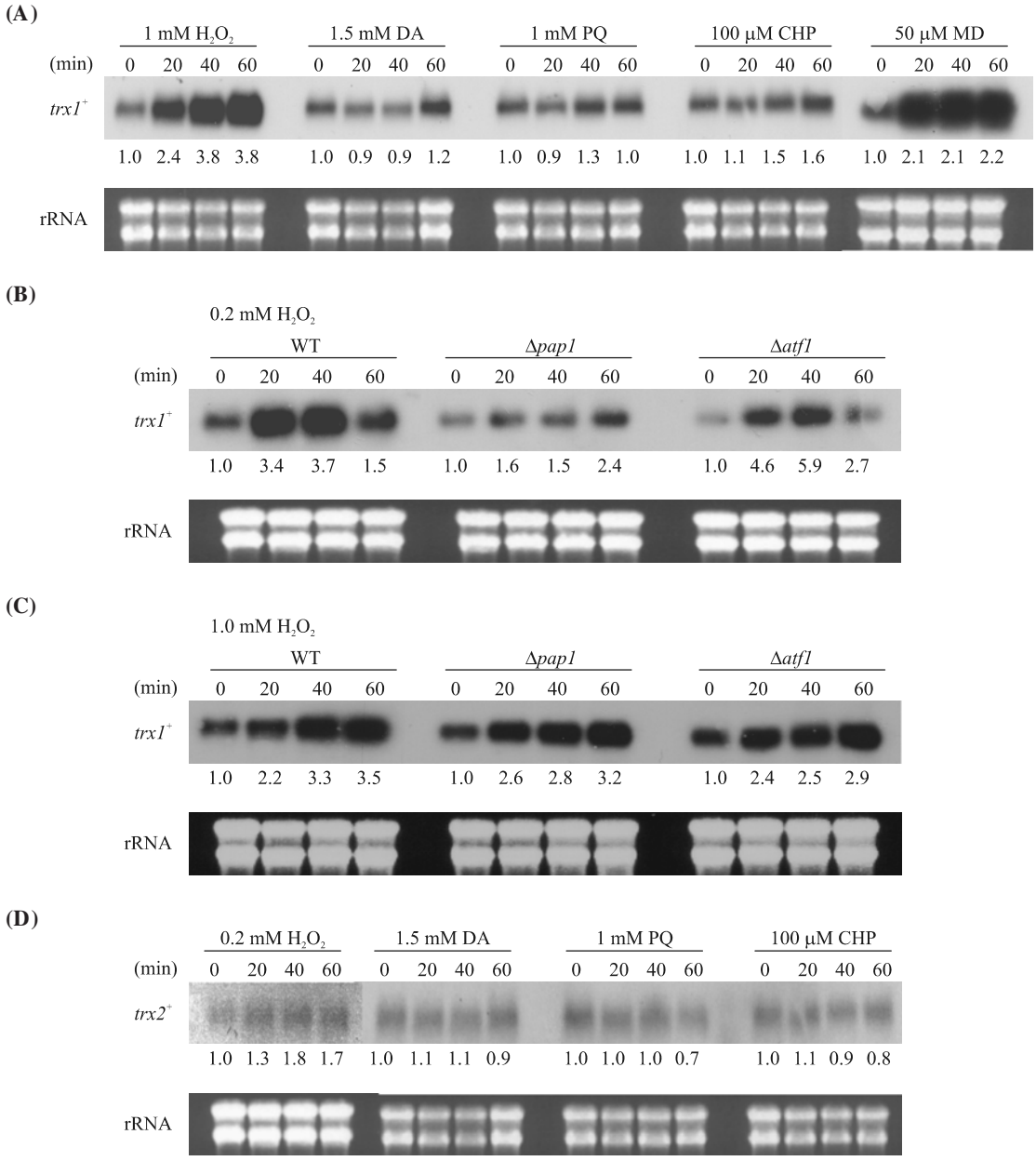

Fig. 2. Induction of *trx1*$^+$ or *trx2*$^+$ mRNA under oxidative stress conditions. (A) Time course of induction of *trx1*$^+$ mRNA. The total RNA was prepared from exponentially grown wild type (ED665) cells either untreated or treated with 1 mM H_2O_2, 1.5 mM diamide (DA), 1 mM paraquat (PQ), 100 μM cumene hydroperoxide (CHP), or 50 μM menadione (MD) for 20, 40, or 60 min. Northern blot analysis was performed with *trx1*$^+$ gene probes. Representative blots were shown with relative signal intensities compared with the untreated sample (1.0) indicated at the bottom. (B, C) Effect of *pap1* and *atf1* mutation on *trx1*$^+$ induction. RNA was prepared from exponentially grown wild type, Δ*pap1* (TP108-3c) and Δ*atf1* (KS1497) cells treated with 0.2 mM (B) or 1.0 mM (C) H_2O_2 for indicated time lengths. (D) Time course of induction of *trx2*$^+$ mRNA. RNA was prepared from exponentially grown wild type cells treated with 0.2 mM H_2O_2, 1.5 mM DA, 1 mM PQ, or 100 μM CHP for indicated time. A representative blot was shown with relative intensities quantified.

was not much induced by oxidants. Only H_2O_2 increased *trx2*$^+$ transcripts to about 2-fold (Fig. 2D). The induction of *trx2*$^+$ by H_2O_2 depended primarily on Pap1 with less dependence on Atf1 (data not shown). The consensus Pap1 recognition sequences (TTACGTAA and TTAGTAA with conserved palindromic bases underlined; Fujii *et al*., 2000) with one-base mismatch were found in the upstream of *trx1*$^+$ and *trx2*$^+$ genes within 550 bp from the start codon, supporting the observation. The differential gene expression of these two Trx genes in response to various stresses may have some relevance with their different sub-cellular localization and function.

Effect of *Trx1* on the activity of Pap1

In *S. cerevisiae*, the double disruption of *TRX1 TRX2* caused constitutive activation of Yap1, an oxidant-sensitive transcrip-

tional regulator, revealing that thioredoxin functions as a negative regulator of Yap1 nuclear localization and transcriptional activation (Izawa *et al*., 1999). We examined whether the activity of Pap1, a Yap1 orthologue in *S. pombe*, is modulated by Trx1 by monitoring expression of Pap1-regulated genes. Transcripts from the *ctt1*$^+$ gene encoding a catalase (Nakagawa *et al*., 2000) and the *trr1*$^+$ gene encoding a thioredoxin reductase (Kang *et al*., 2006) were examined by Northern hybridization analysis in the wild type, Δ*trx1* and Δ*pap1* mutant cells. As demonstrated in Fig. 3A, the two genes known to be induced by oxidants in Pap1-dependent manner, were induced by 0.2 mM H_2O_2 in the wild type but not in Δ*pap1* mutant as expected. We found that in Δ*trx1* cells the basal (unstressed) level of the *ctt1*$^+$ and *trr1*$^+$ transcripts were all elevated. In addition, the transient nature of the induction with decreased expression at 60 min post-treatment was compromised in the Δ*trx1* mutant. This suggests that Trx1 contributes to maintaining Pap1 in its reduced state before and after oxidant treatment. The absence of Trx1 did not increase the amount of ROS in the cell as judged by no increase in protein carbonylation in Δ*trx1* (data not shown). Therefore, it is most likely that Trx1 specifically affects the thiol-disulfide status of Pap1, as implied from the role of thioredoxins on Yap1 in *S. cerevisiae* (Kuge *et al*., 2001). Interestingly, while the *trr1*$^+$ transcription was not induced by 1.5 mM DA in the wild type cells, it was induced by DA-in Δ*trx1* cells (Fig. 3B).

Trx1 is required for sulfur assimilation

Trx1 is dispensable for growth in complex medium such as YES medium. However, we found that Δ*trx1* cells did not grow on minimal EMM plates (Fig. 4A) or in liquid minimal media (data not shown). In other organisms, it has been reported that Trxs or Grxs serve as electron donors to 3'-phosphoadenosine-5'-phosphosulfate (PAPS) reductase in cysteine biosynthetic pathway (Fig. 4B; Masutani and Yodoi, 2002). By adding cysteine to media, Δ*trx1* mutants resumed growth in EMM plate (Fig. 4A). Not only cysteine but also glutathione or *N*-acetyl-L-cysteine could recover the growth defect of Δ*trx1* in minimal medium because they could be decomposed and supply cysteine in the cell (data not shown). To confirm whether the cysteine auxotrophy resulted from inactivation of PAPS reductase, we added sulfite (Na_2SO_3), the product of PAPS reductase, to EMM medium, and found that Δ*trx1* mutant grew as well as the wild type (Fig. 4A). In *E. coli*, not only Trxs but also Grxs reduce PAPS reductase (Russel *et al*., 1990). However introduction of multicopy *trx2*$^+$, *pgr1*$^+$, or *grx1*$^+$ into Δ*trx1* mutant did not overcome the cysteine auxotrophy of Δ*trx1* (data not shown). This implies that neither mitochondrial thioredoxin, nor cytosolic glutaredoxin substitute for the role of Trx1 in supporting cysteine biosynthesis in *S. pombe*.

Conclusions

In this study we demonstrated that Trx1 serves as a major defense system against oxidative, heat, and salt stress in *S. pombe*. It could contribute by reducing disulfide bonds of oxidized proteins, and by supporting oxidant-scavenging proteins such as thioredoxin-dependent peroxidase Tpx1 (Jara *et al*., 2007). It also functions as a support system for cysteine synthesis, by serving as a sole electron donor for

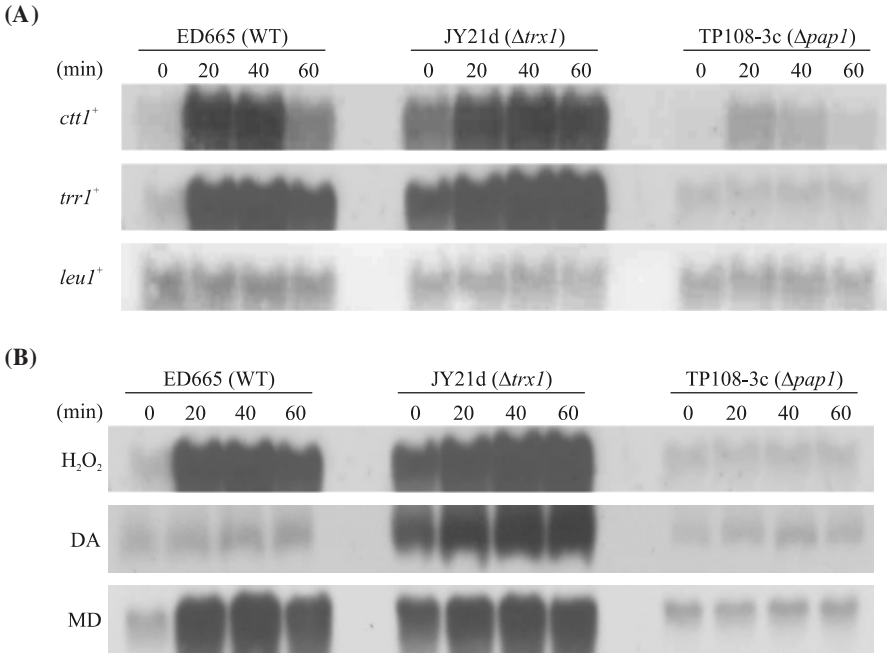

Fig. 3. Effect of disruption of *trx1* on the expression of Pap1-regulated genes. (A) Total RNA was isolated from the wild type and different mutant cells treated with 0.2 mM H_2O_2 for 20, 40, and 60 min at early exponential phase. Northern blot analysis was performed with probes specific for *ctt1*$^+$, *trr1*$^+$, and *leu1*$^+$ genes. (B) Induction of *trr1*$^+$ transcripts by different oxidants in different genetic background. Cells were treated with 0.2 mM H_2O_2, 1.5 mM diamide, and 50 μM menadione. Northern blot analysis was performed with *trr1*$^+$-specific probe.

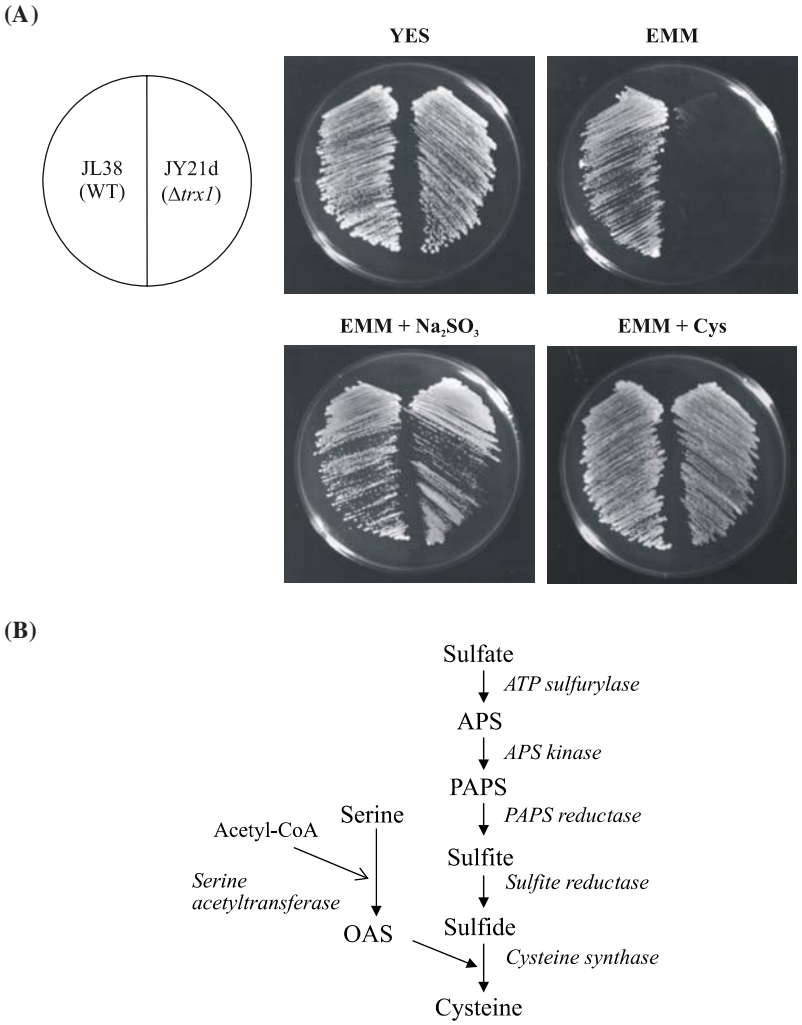

Fig. 4. Requirement of Trx1 for cysteine biosynthesis. (A) Auxotrophy of Δ*trx1* mutant. The wild type and Δ*trx1* (JY21d) cells were streaked on YES, EMM, or EMM plates supplemented with 1.4 mM cysteine or 2 mM Na_2SO_3. Photos were taken after incubation at 30°C for 4 days. (B) Biosynthetic pathway for cysteine. APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; OAS, *o*-acetylserine.

PAPS reductase. Expression of Trx1 is induced at the level of transcription in response to oxidants, and this induction is primarily mediated through Pap1. The nuclear localization and activation of Pap1 is regulated by oxidation of N-terminal and C-terminal cysteine-rich domain (Toon and Jones, 1998; Kudo *et al*., 1999). This activation is mediated through Tpx1, a 2-Cys peroxiredoxin (Prx) (Bozonet *et al*., 2005; Vivancos *et al*., 2005). In this study, we suggest that Trx1 inactivates Pap1 by reducing its disulfide bonds. Since *trx1*$^+$ expression itself is induced by oxidative stress in a Pap1-dependent manner, the reduction of Pap1 by Trx1 constitutes a negative feedback loop.

Acknowledgements

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