A yeast two-hybrid knockout strain to explore thioredoxin-interacting proteins in vivo

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All organisms contain thioredoxin (TRX), a regulatory thiol-disulfide protein that reduces disulfide bonds in target proteins. Unlike animals and yeast, plants contain numerous TRXs for which no function has been assigned in vivo. Recent in vitro proteomic approaches have opened the way to the identification of >100 TRX putative targets, but of which none of the numerous plant TRXs can be specifically associated. In contrast, in vivo methodologies, including classical yeast two-hybrid (Y2H) systems, failed to reveal the expected high number of TRX targets. Here, we developed a yeast strain named CY306 designed to identify TRX targets in vivo by a Y2H approach. CY306 contains a GAL4 reporter system but also carries deletions of endogenous genes encoding cytosolic TRXs (TRX1 and TRX2) that presumably compete with TRXs introduced as bait. We demonstrate here that, in the CY306 strain, yeast TRX1 and TRX2, as well as Arabidopsis TRX introduced as bait, interact with known TRX targets or putative partners such as yeast peroxiredoxins AHP1 and TSA1, whereas the same interactions cannot be detected in classical Y2H strains. Thanks to CY306, we also show that TRXs interact with the phosphoadenosine-5-phosphosulfate (PAPS) reductase MET16 through a conserved cysteine. Moreover, interactions visualized in CY306 are highly specific depending on the TRX and targets tested. CY306 constitutes a relevant genetic system to explore the TRX interactome in vivo and with high specificity, and opens new perspectives in the search for new TRX-interacting proteins by Y2H library screening in organisms with multiple TRXs.

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Abbreviations: t-BuOOH, tert-butyl hydroperoxide; His-TAC, His-Tag affinity chromatography; MetSO, methionine sulfoxide; TRX, thioredoxin; Y2H, yeast two-hybrid; MSRA, methionine sulfoxide reductase; MET16, PAPS reductase.

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in vitro, especially in plants, and second in opening new perspectives for the systematic in vivo search for new TRX-interacting proteins in all organisms.

Materials and Methods
Strains and Media. The yeast strain EMY63 (32) was used for both phenotypic comparisons and production of recombinant TRXs. The Y2H strains YRG2 (Stratagene) and YM954 (33) were used for deletion of TRX1 and TRX2, respectively. Construction of strain CY306 was achieved by the long flanking homology method (34) and is described in Supporting Materials and Methods, which is published as supporting information on the PNAS web site. Chemicals [H2O2, tert-butyl hydroperoxide (t-BOOH), diamide, Met, and MetSO] were purchased from Sigma. All media are detailed in Supporting Materials and Methods.

Molecular Cloning. Yeast and Arabidopsis ORFs, primers, and MuI.NaeL.BglIII (MB) adaptor used in this study are given in Table 2, which is published as supporting information on the PNAS web site. PCR products were obtained by using the long expand template enzyme (Roche Applied Science). Mutation of cysteine residues was obtained by using the Site-Directed Mutagenesis kit (Stratagene). All DNA fragments were systematically checked by sequencing.

TRX His-Tagging and Purification of TRX/Target Complexes. TRX baits were cloned into a modified version of the pFL61 shuttle vector (ref. 35 and Supporting Materials and Methods) and were separately introduced in EMY63 strain for production of recombinant His-tagged TRX baits. Cultures, protein extractions, and complex purifications were performed as described (26) by using 1 ml of His-Bind N2+ resin (Novagen) per liter of cell cultures (OD600 ranging between 1.4 and 2). Protein complexes were treated immediately or frozen at −80°C.

Protein Analysis. Extraction of total proteins for immunoblot analyses are described in Supporting Materials and Methods. All proteins were quantified by using a protein assay kit (Bio-Rad). Proteins for mass spectrometry were concentrated by using frozen acetone and, once lyophilized, were solubilized in 500 μl of solubilization buffer [9 M urea/4% CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate)/0.5% Triton X-100/0.5% immobilized pH-gradient (IPG) buffer (Amersham Pharmacia)/3 mM tributyl phosphin]. Proteins (300 μg) were first separated along linear IPG strips (pH 3–10, 18 cm long) by using an IPGphor apparatus (Amersham Pharmacia Biosciences) and further treated as described (36). Second dimensions were performed in 13% polyacrylamide/SDS gels. Proteins were stained by colloidal Coomassie and digested by trypsin, and individual spots were analyzed by MALDI-TOF (BIFLEX III, Bruker, Billerica, MA) at the Montpellier LR Genopole (France).

Two-Hybrid Experiments. All experiments were performed in the yeast reporter strains YRG2 (Stratagene) and CY306 (this study). cDNAs encoding AtTRXh3ser62, AtTRXh3ser42, the yeast TRX1ser33 and TRX2ser34 are available in our laboratory and were cloned at EcoRI-BamHI sites of pGBKT7 (Clontech). Target cDNAs [AHPI, TSA1, methionine sulfoxide reductase (MSRA), and MET16] were obtained by PCR amplification on yeast genomic DNA by using restriction site-containing primers (Table 2), and cloned into pGADT7 (CLONTECH) at corresponding sites. AtTDXA (270–380) and SSB2 constructs are from ref. 37. Double transformants in YRG2 and in CY306 strains were selected as cells growing in the absence of leucine and tryptophan, and in the presence of Met and genicin for CY306 strain. Cells bearing interacting proteins were selected on media lacking leucine, tryptophan, and histidine, but containing Met and 20 mM 3-aminotria-
TRX2 are also hypersensitive to oxidant molecules such as H$_2$O$_2$ (32) and t-BOOH (39), we checked that CY306, like EMY63 mutant, is unable to grow on plates containing H$_2$O$_2$ (Fig. 1B). Both strains were also unable to grow on plates containing 0.8 mM t-BOOH (Fig. 1B). Interestingly, the ym954trx2A mutant was also unable to grow, suggesting that TRX1 cannot compensate the function of TRX2 in the case of reduction of alkyl hydroperoxides by TRXs. Finally, the CY306 mutant showed resistance to diamide and sensitivity toward DTT (Fig. 1C), similarly to trx2A (32) and trx1A trx2A (40) mutants. Altogether, these results indicate that the CY306 strain is true trx1A trx2A mutant regarding phenotypes.

CY306 trx1A trx2A Yeast Strain Is a Y2H Tool That Reveals Thioredoxin Targets in Vivo. To evaluate the efficiency of the Y2H CY306 strain to reveal interaction involving thioredoxins, binary interactions between TRXs and putative targets were assayed in both the mutant CY306 and the wild-type YRG2 strains. All TRXs used as bait carried a mutation of the second cysteine of the TRX active site into a serine (TRX1ser33 and TRX2ser34), in accordance with the ability of mutated TRXs to establish stable mixed disulfide complex in vivo (26). Four different targets were chosen, either because they had already been described as TRX targets by other means, or because of strong presumption that they are TRX targets.

The first protein tested was the thioredoxin-dependent peroxidase AHP1, a yeast protein involved in alkyl hydroperoxide detoxification (39, 41). Biochemical and genetic evidence indicates that AHP1 is a TRX target (26) and is consistent with sensitivity of trx1A trx2A yeast mutants to H$_2$O$_2$ and t-BOOH. However, a previous study using classical Y2H systems failed to reveal a TRX/AHP1 interaction (26). Fig. 2A shows that, in the mutant strain CY306, a faint interaction could be detected between TRX1ser33 and AHP1, and between TRX2ser34 and AHP1 to a much greater extent. We also tested the interaction between both TRXs and TSA1, a thiol-specific antioxidant also involved in hydroperoxide detoxification and that requires the TRX/NADPH thioredoxin reductase (NTR) reductase system (5). A strong interaction was obtained with TRX2ser34 only in the CY306 strain (Fig. 2B), whereas no interaction was found with TRX1ser33. None of the two TRXs interacted with both targets in the YRG2 strain.

Another putative TRX-interacting protein that was assayed is the yeast MSRA. MSRA is another antioxidant enzyme that exerts its protection against oxidative stress by maintaining a low level of oxidized Met, either in protein-bound or free amino acid form (42). In vivo and in vitro analyses of yeast (38) and E. coli (43, 44) TRX mutants indirectly suggested that TRX is the reductase that reactivates methionine sulfoxide reductase (38, 43, 44), which is consistent with the inability of trx1A trx2A yeast mutants to grow on plates containing MetSO as the sole Met source (ref. 38 and this work). As shown in Fig. 2C, cells bearing either MSRA/TRX1ser33 or MSRA/TRX2ser34 were able to grow on selective medium only in the strain CY306, thus confirming that both TRXs directly interact with MSRA in vivo.

All trx1A trx2A yeast mutants described in the literature exhibit a Met auxotrophy, due to the inability to assimilate inorganic sulfur (32, 40). In yeast, TRX is required in sulfur assimilation (7, 8) and is supposed to ensure the reduction of a conserved cysteine in the PAPS reductase (MET16), a key enzyme required for Met biosynthesis (45). We actually found a strong interaction between MET16 and TRX1ser33 in CY306 strain (Fig. 2D), but a weaker but significant interaction with TRX2ser34 could also be demonstrated in this strain. In both cases, such interactions could not be observed in the wild-type strain YRG2.

All interactions visualized using His3 reporter gene under Gal4 control were confirmed by using LacZ reporter gene, for both the presence and intensity of interaction (data not shown). No interaction (using both reporter genes) was observed when nonmutated forms of TRX were used as bait (data non shown).

CY306 trx1A trx2A Yeast Strain Reveals Specific and Low-Abundant TRX Targets. To compare the efficiency of the CY306 strain and the TRX-specific Y2H system with other in vivo approaches, we performed a series of purifications of TRX-target complexes established in vivo after production of His-Tagged mutated TRXs in the yeast mutant Emy63 trx1Δ trx2Δ. Experiments were performed by using yeast TRX1ser33 and TRX2ser34 as bait, but also Arabidopsis AtTRXh2ser62 and AtTRXh3ser42.

Among the targets identified by MALDI-TOF (Fig. 3), we confirmed AHP1, TSA1, and MET16 as putative targets. Except for the case of AtTRXh3ser42, which did not allow TSA1 and MET16 purification, these targets were always isolated (reproductive isolation), independent of the bait used (Table 1). This apparent lack of specificity contrasted with that observed with the CY306 Y2H system (Table 1 and Fig. 2). Of particular interest was the nonspecific purification of TSA1 using all baits in the His-TAC method, although it only interacted with the yeast TRX2ser34 in CY306 (Fig. 2B). In this case, the Y2H result was more consistent with the putative function of the yeast TRX2 in response to oxidative stress produced by alkyl hydroperoxides, as shown by phenotypic analyses of trx1A trx2Δ yeast mutants (Fig. 1B and ref. 41). This difference of specificity between the two targeting systems was also shown when plant TRXs were used as bait. AtTRXh2ser62 always captured AHP1, TSA1, and MET16 by the His-TAC method (Fig. 3), although it interacted with MET16 only by the CY306 Y2H approach (Table 1). Only the result obtained by using CY306 is consistent with the ability of AtTRXh2 to complement Emy63 trx1A trx2Δ mutant for Met auxotrophy by its ability to reduce the
Fig. 3. Bidimensional electrophoresis of TRX/targets complexes established in vivo in EMY63 trx1Δ trx2Δ cell culture. The different TRXs used as baits are indicated under the corresponding panels. Numbers refer to identified proteins/orf as follow: 1, alcohol dehydrogenase/YLO086C; 2, glyceraldehyde-3-phosphate dehydrogenase, isozyme 1/YLR192C; 3, glyceraldehyde-3-phosphate dehydrogenase, isozyme 1/YLO052W; 4, thioredoxin reductase/YDR353W; 5, MET16/YPR167C; 6 and 12–14, eukaryotic initiation factor 4E/YEL071W; and 16, D-lactate dehydrogenase/YEL071W. The pH gradient is indicated above the panels. Size of markers is indicated in kDa on the left of each panel. Control experiments using nonmutated TRX exhibited only spots corresponding to the baits used (data not shown). Results shown here are the means of four independent experiments for each TRX bait.

In the His-TAC approach, we also found that AHP1, TSA1, and MET16 were always similarly isolated (2D-spot density). On the opposite, the CY306 strain not only allowed the detection of TRX/target interactions in a specific manner, but also revealed different levels of interaction depending on the partners analyzed (Table 1).

Finally, except for the mid-abundance MET16 protein, all of the targets identified by the His-TAC method were high-abundance proteins (Fig. 3), although we did not identify low-abundance proteins including the MSRA protein among the 2D-spots. In contrast, a Y2H interaction could be detected in CY306 between proteins including the MSRA protein among the 2D-spots. In targets identified by the His-TAC method were high-abundance populations of markers is indicated in kDa on the left of each panel. Control experiments using nonmutated TRX exhibited only spots corresponding to the baits used (data not shown). Results shown here are the means of four independent experiments for each TRX bait.

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Altogether, these results suggest that, even if the His-TAC is very helpful in the isolation of TRX targets, the CY306 Y2H system is far more sensitive for TRXs target recognition. The CY306 Y2H system provides information that is consistent with functional complementation data (38) and the specificity and sensitivity values that are lacking in the His-TAC system developed here.

MET16/PAPS enzyme, whereas it is unable to complements EMY63 for sensitivity to oxidative stress (38).

We finally investigated the ability of the CY306 strain to reveal critical residues such as cysteines involved in TRX interaction with its target. We took as an example the MET16 enzyme that contains two cysteine residues: C112, which does not possess any catalytic activity (46), and C245 in the consensus sequence KxECG(L/I), which is required for MET16 activity (47). Before this work, the identity of the cysteine residue targeted by TRX in MET16 was unknown. Two mutated MET16C112S and MET16C245S were then constructed in pGAD77 and further assayed for interaction with both TRX1ser33 and TRX2ser34. As shown in Fig. 4, the C112S mutation in the MET16 protein did not affect its interaction with TRX1ser33 and TRX2ser34 because CY306 growth remained similar to that observed for the wild-type protein. In contrast, the C245S mutation in MET16 drastically abolished its binding to both TRXs as revealed by the absence of CY306 growth. This result first confirmed that C245 is the essential cysteine for MET16 interaction with TRXs (47). Second, this experiment demonstrated that the CY306 strain is a functional and reliable tool to discriminate cysteines targeted by TRXs in vivo.

**Table 1. Comparative analysis between His-TAC and Y2H methods to efficiently reveal specific TRX/target complexes in trx1Δtrx2Δ yeast mutants**

<table>
<thead>
<tr>
<th>Targets</th>
<th>Affinity chromatography*</th>
<th>Yeast two-hybrid†</th>
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<tr>
<td></td>
<td>TRX1</td>
<td>TRX2</td>
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<td>AHP1</td>
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<td>TSA1</td>
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<td>MET16</td>
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<tr>
<td>MSRA</td>
<td>Nl</td>
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*Plus signs (+ + + + +), spot density as observed on 2D gels. Nl, the corresponding target was not identified among the 2D spots analyzed.
†Plus signs (+ + + + +), the level of two-hybrid interactions; –, no interaction occurred between partners tested; + / –, a faint but reproducible interaction.

The CY306 strain allows detection of the essential cysteine residue in the PAPS reductase that is required for TRX interaction. Both the TRX1ser33 or TRX2ser34 baits were tested for two-hybrid interaction with the two variants MET16C112S and MET16C245S of the MET16 enzyme. Cells were grown and spotted as described in Fig. 1. Results shown here are the means of three independent experiments.

**Discussion**

Proteins rarely work by themselves and almost all require other molecules to execute their functions. TRXs are among such pro-
proteins that were historically shown to be involved in substrate reduction and regulation of enzymes in physiological conditions. In the last few years, extended efforts have been devoted to large-scale searches for potential TRX targets in various organisms. Thanks to in vitro approaches (affinity chromatography and proteomics), several nonexhaustive lists of putative TRX targets have been established, deciphering new pathways and metabolic functions involving TRXs (11). Because of a real need to go further in the analysis of TRX/target complex specificity, we describe here a tool consisting of a yeast strain devoted to the study of binary protein/protein interactions and Y2H screenings in vivo, specifically designed for the identification and analysis of TRX targets. We propose that this tool will be of great help, first to assign specific targets to specific TRXs, and second to isolate new and/or low-abundant candidates for reduction by TRXs in vivo.

A Two-Hybrid System for Thioredoxins. Numerous two-hybrid systems have been described, most of them using bacterial or yeast cells. The Y2H system was selected in our strategy for several reasons. Yeast combines an ease of manipulation comparable with that of bacteria with the characteristics of eukaryotic cells, such as posttranslational modifications. The Y2H system is adaptable to most kinds of baits and targets from any organelle or organism, and allows not only the study of binary interaction between two proteins, but also the search for new partners in DNA libraries. Interactions occur in vivo, which distinguishes this approach from other protein-targeting methods, and can be performed between two proteins from different organisms. Unfortunately, when we first used commercial Y2H systems to search for TRX targets using DNA libraries, using yeast or plant TRXs as bait, very few targets were isolated, in contrast to the numerous putative targets isolated by using in vitro proteomics (19, 20, 48). Even Y2H binary interactions involving TRX targets isolated by another in vivo method failed in classical Y2H yeast strains (26). The poor efficiency of classical Y2H systems to reveal TRX targets has been observed in previous library screenings (28–30, 49), and only a few TRX partners have been isolated up to now (27–31). Some TRX-interacting partners, such as ASK1 (50), the S Locus receptor kinase SRK (51), and the C9-protein (52) were also revealed, but in which interacting partners, such as ASK1 (50), the S Locus receptor kinase SRK (51), and the C9-protein (52) were also revealed, but in which pathways involving such dithiol proteins. Nevertheless, some limits inherent to both methodologies have been brought to light. Concerning affinity chromatography, one may question the unspecific binding on affinity column of proteins unrelated to TRX function, the absence of clear results concerning specificity of the targets regarding cellular compartments, and the variety of proteins captured by the different dithiol isoforms (21). In our His-TAC system, we also observed low specificity in the targets isolated, probably due to the use of a multicycle shuttle vector for production of recombinant TRX bait in vivo. On the other hand, using CY306 strain, we found that yeast TRX1 interacts preferentially with the yeast PAPS enzyme, although TRX2 preferentially interacts with peroxidases involved in detoxification toward oxidant molecules. Interestingly, both TRX1 and TRX2 interact with the TRX-dependent AHP1, whereas only TRX2 interacts with the thiol peroxidase TSA1. The CY306 Y2H system demonstrates that a specificity in TRX/target recognition can be revealed and opens perspectives in deciphering the true functions of the numerous TRX isoforms. Many putative targets have already been isolated, even with low specificity, and could be now tested for Y2H interaction with the different TRX isoforms originated from the same organism.

CY306 Strain Is a New Tool for the Identification of Residues Required for TRX/Target Interactions. Interestingly, all interactions observed in this study were obtained with TRX baits carrying only a Cys-to-Ser mutation on the second cysteine of the CXXC active site, confirming the requirement of such mutation to establish stable intermediate complexes between the TRX and targets tested here. Nevertheless, several putative targets already isolated by in vitro means do not contain any conserved cysteines (18, 60). Other targets contain cysteines that are not conserved in other organisms (18). These observations raise the question as to how TRX binds to such targets. We have shown that CY306 is able to reveal the C245 as the amino acid targeted by TRX in MET16 protein. The same analysis could now be performed with other putative targets for which a question mark still remains about being true TRX partners, by using both wild-type and mutated TRXs as bait to answer whether cysteines of both partners are required for interaction.

Searching for Low-Abundant and/or New TRX-Interacting Proteins. One of the realities of large-scale in vitro proteomics is the low efficiency to reveal low-abundance protein targets. In our His-TAC approach, almost all of the putative targets isolated by the His-TAC approach were highly abundant proteins. Despite several purification experiments, we always failed to isolate low-copy targets, such as the MSRA protein, probably because the more abundant targets such as AHP1 quench the foreign TRX bait. On the other hand, Y2H systems are usually efficient to detect low-copy-number proteins, such as transcription factors, because interactions are required for cell life. By using the CY306 Y2H strain, we obtained an interaction between MSRA and both TRX1ser33 and TRX2ser34, as well as with AtTRXh2ser62 and AtTRXh3ser42, suggesting that CY306 is better adapted to reveal low-abundance partners. This result was confirmed by the isolation of a low-abundant transcription factor of the TFIID complex from a yeast cDNA library screening using the Y2H
Concluding Remarks. Deciphering the entire TRX interactome is now required for our understanding of their functions and interpretation of sequenced genomes. Plants are probably the most complex organisms regarding TRX gene families, with >40 TRX-related proteins (e.g., for Arabidopsis). Up to now, we have been unable to assign a specific TRX to a specific protein among the numerous putative targets revealed by proteomics. With the availability of CY306, we hope to overcome this problem. The next step involves starting a Y2H for all of the Arabidopsis TRXs and putative partners already isolated by different means from this plant. Combined with proteomics, the CY306 tool should be of great help in exploring the composition of TRX-containing complexes and in proceeding to TRX biological functions in plants as well as in other organisms.

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