Activation of the Oxidative Stress Regulator PpYap1 through Conserved Cysteine Residues during Methanol Metabolism in the Yeast Pichia pastoris

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The methylotrophic yeast Pichia pastoris can grow on methanol as sole source of carbon and energy. The first reaction in yeast methanol metabolism, catalyzed by an abundant peroxisomal enzyme, alcohol oxidase, generates high levels of H$_2$O$_2$, but the oxidative stress response during methanol metabolism has not been elucidated. In this study, we isolated the Yap1 homolog of P. pastoris (PpYap1) and analyzed the properties of a PpYAP1-disruption strain. The PpYap1 transcription factor is activated after exposure to various reactive agents, and therefore functions as a regulator of the redox system in P. pastoris. We have also identified PpGPX1, the unique glutathione peroxidase-encoding gene in P. pastoris whose expression is induced by PpYap1. PpGpx1, but not the ScTsa1 or SpTpx1 homolog PpTs1, functions as a H$_2$O$_2$ sensor and activates PpYap1. This study is the first demonstration of a yeast Yap1 family protein activated during conventional metabolism.

Key words: redox regulation; oxidative stress; methylotrophic yeast; methanol metabolism

The methylotrophic yeasts, e.g., Pichia pastoris, Candida boidinii, and Hansenula polymorpha, can grow on methanol as sole source of carbon and energy. These yeasts have a highly specialized metabolic pathway for methanol-metabolism that is partly compartmentalized in peroxisomes. An abundant peroxisomal enzyme, alcohol oxidase (Aox), catalyzes the oxidation of methanol, resulting in the formation of a great deal of hydrogen peroxide (H$_2$O$_2$). Hence the peroxisomal matrix and membranes are assumed to be exposed to a high level of reactive oxygen species (ROS), which can also cause damage to all other cellular constituents, nucleic acids, proteins, lipids, etc. Therefore, as in other ROS generating organelles, such as mitochondria, peroxisomes are assumed to have defensive enzyme activities against ROS toxicity. The methylotrophic yeasts have two peroxisomal antioxidant enzymes, catalase and peroxisomal glutathione peroxidase (Pmp20), which decompose H$_2$O$_2$ and alkyl hydroperoxides respectively. In P. pastoris, disruption of PpPMP20 caused a more severe growth defect on methanol media than deletion of the catalase gene PpCTA1, as was the case for their C. boidinii homologs, implying a high level of ROS formed upon the generation of H$_2$O$_2$ during methanol conversion. While these antioxidant enzymes have been well-characterized, the stress response mechanism during yeast methanol metabolism has yet to be elucidated.

In the budding yeast Saccharomyces cerevisiae, a central regulator of the response to oxidative stress is the transcriptional factor ScYap1. Upon exposure of yeast cells to increased levels of ROS, ScYap1 rapidly accumulates in the nucleus, where it regulates the expression of numerous antioxidant defense genes. In unstressed cells, ScYap1 is freely imported into and exported from the nucleus. In cells exposed to oxidative stress, nuclear export is arrested because ScYap1 can no longer interact with the conserved nuclear export signal (NES) that harbors the NES generating organelles, such as mitochondria. Therefore, as in other ROS generating organelles, such as mitochondria, peroxisomes are assumed to have defensive enzyme activities against ROS toxicity. The methylotrophic yeasts have two peroxisomal antioxidant enzymes, catalase and peroxisomal glutathione peroxidase (Pmp20), which decompose H$_2$O$_2$ and alkyl hydroperoxides respectively. In P. pastoris, disruption of PpPMP20 caused a more severe growth defect on methanol media than deletion of the catalase gene PpCTA1, as was the case for their C. boidinii homologs, implying a high level of ROS formed upon the generation of H$_2$O$_2$ during methanol conversion. While these antioxidant enzymes have been well-characterized, the stress response mechanism during yeast methanol metabolism has yet to be elucidated.

In this study, in order to investigate the physiological stress response during yeast methanol metabolism, we isolated the P. pastoris homolog of YAP1 (PpYAP1) and compared its structural characteristics with other yeast Yap1 homologs. We analyzed the role of PpYap1 and its localization in response to H$_2$O$_2$ and other oxidative stress agents and identified PpGpx1 as an upstream activator of PpYap1. Finally, the cysteine residues...
required for growth during externally applied oxidative stress and during methanol metabolism were identified within PyYap1. Our results indicate that PyYap1 not only plays a critical role in oxidative stress defense analogously to other Yap1 family proteins, but is also essential for growth on methanol. This is the first report that a Yap1 family protein is involved in protection from oxidative stress resulting from normal physiological activity.

Materials and Methods

Strains and media. Most of the experiments were performed with wild-type strain PPy12 (arg4 his467) and its isogenic derivatives. Escherichia coli DH10B was routinely used in plasmid propagation. The P. pastoris strains were grown on YNB medium (0.67% yeast nitrogen base without amino acids), supplemented, when required, with appropriate amino acids (200 μg/ml for arginine, 200 μg/ml for histidine) or Zeocin (Invitrogen, Carlsbad, CA) (50 μg/ml). The following media were used as the carbon source in YNB medium: 2% (wt/vol) glucose (SD), and 2% (vol/vol) methanol (SM). The initial pH of the medium was adjusted to 6.0. Yeasts were cultivated aerobically at 28°C, with reciprocal shaking, and their growth was followed by measuring the optical density at 610 nm (OD610), and the reciprocal shaking, and their growth was followed by measuring the optical density at 610 nm (OD610).

Expression of PyYap1 and its derivatives in P. pastoris. To visualize the localization of PyYap1, a strain expressing a DsRed monomer fluorescent protein (mRed)-PyYap1 fusion protein was constructed as follows: First, using genomic DNA as a template, a 1.4-kb SpeI-Sphi fragment containing the PyYap1 coding region and a 0.8-kb NdeI-EcoRI fragment with the PyYap1 promoter (P<sub>Pyap1</sub>) region were amplified by PCR with primer sets PyYap1-SpeI-Fw/PyYap1-Sphi-Rv respectively. Then, a 0.7-kb PCR fragment from the upstream region of the Ppyap1 gene were amplified by PCR with primers Ppyap1-SpeI-Fw/Ppyap1-Sphi-Rv, using vector pDsRed-Monomer-N1 (Clontech Laboratories, Inc., Mountain View, CA) as a template. The PCR products were recombined with mRed-EcoRI-Fw and mRed-SpeI-Rv, using vector pDtsa1. After it was linearized with XhoI, pDtsa1 was transformed into the wild-type strain E. coli DH10B was routinely used in plasmid propagation. The P. pastoris strains were also derived by electroporation. Zeocin-resistant colonies were selected on SD medium supplemented with arginine, histidine, and Zeocin. Disruption of the Ppyap1 gene was confirmed by Southern blot analysis using Ndel-digested genomic DNA of transformants and a 1.0-kb XhoI-SacII PCR fragment from the upstream region of the Ppyap1 gene as probe. In a similar way, the Pyap1Δ and Pyap1Δ strains were also derived with PyYap1- and PyGPX1-disruption vectors. Primer pairs PyYap1-up-NotI/Ppyap1-up-PstI, PyYap1-down-PstI/Ppyap1-down-XhoI, PyGPX1-up-NotI/PPyGPX1-up-PstI, and PyGPX1-down-PstI/PPyGPX1-down-XhoI (Table 1) were used in amplification of the upstream and downstream regions of the respective genes.

Table 1. Oligonucleotide Primers Used in This Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
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<tbody>
<tr>
<td>PpTSA1-up-XhoI</td>
<td>CCGCTGCAAGATCTCTGCAAGAATGCTAGTT</td>
</tr>
<tr>
<td>PpTSA1-up-SacII</td>
<td>TCCCGGCGTAAGACGATAGATTGAAGAT</td>
</tr>
<tr>
<td>PpTSA1-down-KpnI</td>
<td>GGGTGACCAAGAAATAACGTAATAGTTT</td>
</tr>
<tr>
<td>PpTSA1-down-XhoI</td>
<td>CCGGCTGAGATTTCTTCTTCATAAAGC</td>
</tr>
<tr>
<td>PpYAP1-up-NotI</td>
<td>ATATGATGCTGGCGCGAGTTGATTT</td>
</tr>
<tr>
<td>PpYAP1-up-PstI</td>
<td>AACTGCAAGCTCTGCTGATTCAAGGTTAATTT</td>
</tr>
<tr>
<td>PpYAP1-down-XhoI</td>
<td>CCGGCGCGAGTTGACCTTTATATTCCAGG</td>
</tr>
<tr>
<td>PpGPX1-up-XhoI</td>
<td>CCGGCGCGAGTTGACCTTTATATTCCAGG</td>
</tr>
<tr>
<td>PpGPX1-up-PstI</td>
<td>AACTGCAAGCTCTGCTGATTCAAGGTTAATTT</td>
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<tr>
<td>PpGPX1-down-PstI</td>
<td>AACTGCAAGCTCTGCTGATTCAAGGTTAATTT</td>
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<td>PpGPX1-down-PstI</td>
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The underlined nucleotide sequences are additional restriction enzyme recognition sequences.
Quantitative real-time PCR (qRT-PCR). Cells were grown aerobi-
cally to mid-exponential phase in SD medium with and without
reactive reagents. Total RNAs were isolated from cells with an RNeasy
mini kit (QIAGEN K.K., Tokyo), followed by on-column DNase
digestion. cDNAs were synthesized from 1 m\(\text{g}\) total RNA using
Random Primers (Promega, Madison, WI) and ReverTra Ace (Toyobo
Co., Ltd., Osaka). After reverse transcription for 50 min at 42
\(^\circ\text{C}\), samples were heated for 5 min at 99
\(^\circ\text{C}\) to terminate the reaction, and
0.5 ml of RNase H was added. qRT-PCR was performed in 20-
m\(\text{l}\) mixtures in glass capillary tubes in a LightCycler (Roche Diagnostics
K.K., Tokyo, Japan) using
1 SYBR Premix Ex taq (Takara Bio,
Kyoto) and the primers listed in Table 1, according to the following
parameters: first cycle, 10 min denaturation at 95
\(^\circ\text{C}\); second cycle with
40 repetitions, 95
\(^\circ\text{C}\) 20 s, 55
\(^\circ\text{C}\) 20 s, and 72
\(^\circ\text{C}\) 20 s (all temperature
transitions, 20
\(^\circ\text{C}\)). As a negative control the PCR was done
without ReverTra Ace. The relative abundance of mRNAs was
standardized against the levels for
\(\text{PpACT1}\).

Fluorescence microscopy and image acquisition. \(P.\) pastoris
cells carrying mRed-PpYap1 proteins were incubated to mid-exponential
phase in SD medium. The samples were examined under a fluores-
cence-inverted microscope (IX70; Olympus, Tokyo) equipped with a
UPlan Apo 100 x/1.35 oil iris objective lens using mirror/filter units
U-MWIG (Olympus). Image data were captured with a charged-
coupled device camera (SenSys; Photometrics, Tucson, AZ) using
MetaMorph 6.0, and were saved as Photoshop files (Adobe). The TIFF
image files were optimized for their contrast on Photoshop CS3 and
compiled on PowerPoint (Microsoft).

Results and Discussion

Cloning and characterization of the \(P.\) pastoris \(YAP1\)

In \textit{S. cerevisiae}, the transcriptional regulator ScYap1 responds to oxidative stress caused by \(\text{H}_2\text{O}_2\) or ROS, which in \(P.\) pastoris are generated during normal growth on methanol as sole carbon source.\(^1\) To determine the
role of the \(P.\) pastoris homolog (PpYap1) during methanol metabolism, we cloned the correspond-
ing gene, \(PpYAP1\), identified by BLAST search, with
the ScYap1 sequence against the
\(P.\) pastoris genome
data base. The open reading frame encodes a protein of
461 amino acids with a predicted molecular mass of
50,776 Da. According to sequence similarity analysis
using CLUSTALW, the percentage of amino acids of
\(PpYap1\) identical with
\(S.\) cerevisiae
\(ScYap1\) (650 amino
acids) is 32\%, with
\(C.\) albicans
\(Cap1\) (499 amino
acids) 30\%, and with
\(S.\) pombe
(Pap1) (544 amino acids) 23\% (Fig. 1).

The similarity is remarkably high in the bZIP and the
two CRD regions, which is characteristic of Yap1 family
proteins. The bZIP region (Fig. 1B (1)) consists of a
leucine zipper region involved in Yap1 dimerization in
which two clusters of basic amino acids are connected by
a highly conserved spacer crucial for binding to the
target DNA sequence. The leucine zipper region of PpYap1 contains five leucine residues located at every seven amino acids. The corresponding region of the Yap1 homolog, however, varies at the third leucine position, asparagine in \textit{S. cerevisiae}, alanine in \textit{C. albicans}, and threonine in \textit{S. pombe}. This amino acid is considered to be functionally important, since the DNA-binding activity was reduced when asparagine was replaced with leucine in \textit{S. cerevisiae}.

Two cysteine-rich domains (n-CRD and c-CRD) in the C-terminal region of ScYap1 are important for the response to oxidative stress. Cysteine residues are also present at conserved positions in the n-CRD (C303, C310) and c-CRD (C598, C620, C629) of PpYap1. Inter- and intramolecular disulphide bonds can be formed between these cysteine residues, which are furthermore prone to be blocked by thiol-adducts. The interaction between the n-CRD and c-CRD, established by disulphide bonds and which causes the masking of the NES, is further stabilized by F302 and M306 in ScYap1. These five cysteines and F302 are also conserved in PpYap1, but M306 is replaced with leucine (L237), as in \textit{C. albicans} and \textit{S. pombe}.

\textbf{PpYAP1 is involved in the defense mechanism against various reactive agents}

In order to examine whether PpYap1 functions as an oxidative stress response regulator in \textit{P. pastoris}, the \textit{PpYAP1} gene was disrupted by replacing the open reading frame (ORF) with the Zeocin-resistant gene from pREMI-Z as a selective marker. Based on this \textit{Ppyap1\Delta}, we constructed a strain expressing a PpYap1 protein fused to DsRed at its N-terminus (mRed-PpYap1) under the control of the native \textit{P\textsc{YAP1}} promoter. To determine whether PpYap1 is required for the resistance of \textit{P. pastoris} to reactive agents known to activate ScYap1, \textit{Ppyap1\Delta} and the strain \textit{(mRed-PpYap1/Ppyap1\Delta)} were placed on SD agar plates containing hydroperoxides (H$_2$O$_2$, tBOOH, and CHP), menadione, diamide, or NEM. As shown in Fig. 2A, compared to the wild type, the strain lacking PpYap1 (	extit{Ppyap1\Delta}) was highly susceptible to these reagents, indicating a lack of oxidative stress response. The hypersensitivity of \textit{Ppyap1\Delta} was complemented by the expression of mRed-PpYap1, indicating that mRed-PpYap1p was functional.

\textbf{Nuclear localization of PpYap1 is stress-dependent}

Previous work on \textit{S. cerevisiae}, \textit{S. pombe}, and \textit{C. albicans} indicates that regulation of the Yap1 transcription factors involved oxidant-dependent nuclear localization of the protein. To evaluate the extent to which PpYap1 acts analogously to ScYap1, we determined the localization of PpYap1 after exposure to the compounds mentioned above and to cadmium (Cd) and copper (Cu) (Fig. 2B). In the absence of reactive reagents, mRed-PpYap1 exhibited diffuse cytosolic fluorescence. In cells exposed to the compounds tested, mRed-PpYap1 accumulated in the nucleus. This accumulation was reversible, as after washing out the reactive reagents, mRed-PpYap1 gradually showed a diffuse cytosolic fluorescence again.
not shown). Hence the cellular localization of mRed-PpYap1 is indicative of a stress response. Thus the phenotypes observed show that PpYap1 is a key regulator of the defense of *P. pastoris* against a wide variety of oxidative stresses.

In *S. cerevisiae*, the nuclear localization of ScYap1 in response to oxidative stress resulted in transcriptional activation of numerous genes that are involved in preventing oxidative damage to the cells. We investigated to determine whether PpYap1 would activate the transcription of antioxidant genes in response to H$_2$O$_2$, tBOOH, and diamide (Fig. 2C). Upon treatment with these reactive agents, mRed-PpYap1 localized to the nucleus (Fig. 2B) and activated transcription of the target antioxidant genes (Fig. 2C), indicating that PpYap1 is essential for the regulation of the oxidative stress response.

Although activation of PpYap1 was observed with a variety of oxidative reagents, such as hydroperoxides, superoxide anions, diamide, electrophiles, and metal ions, the activation mechanism is thought to differ from case to case. Menadione is both a superoxide anion (O$_2^-$) generator resulting in the formation of ROS and an electrophile capable of forming adducts to cysteine sulfhydryls, like the electrophile NEM. Diamide is a thiol-oxidizing agent that causes fast conversion of GSH to oxidized glutathione (GSSG), resulting in a GSH/GSSG redox imbalance, and oxidation of thiol groups of proteins that thus lose their potential to form disulfide bonds. Cd, which has thiol reactive properties, has been identified as a non-hydroperoxide Yap1 inducer through modification of its cysteine residues on c-CRD. Transition metal ions, such as Cu and iron, convert H$_2$O$_2$ into highly reactive hydroxyl radicals (·OH).

These compounds are assumed to activate ScYap1 in two possible ways: (1) through intramolecular disulfide bonds that cause a change of conformation that prevents Crm1 access to the NES, a route dependent on peroxide and activation by ScOrp1/ScGpx3 (see below), and (2) by forming adducts to cysteine sulfhydryls in the c-CRD near the NES, preventing binding and thus export by Crm1.

**PpGpx1 is exclusively required for the activation of PpYap1 by H$_2$O$_2$**

Activation of ScYap1 by H$_2$O$_2$ appears distinct at the molecular level from its activation by NEM, since H$_2$O$_2$ does not modify sulfhydryl groups directly in vivo. In *S. cerevisiae*, the glutathione peroxidase-like enzyme ScOrp1/ScGpx3 acts as the actual sensor of H$_2$O$_2$ and transduces this signal into ScYap. Another scheme has been reported for *S. pombe*: Tpx1, the *S. pombe* homolog of ScTsa1, is an upstream component of the Pap1 pathway. These observations invite the question whether there is an upstream activator of PpYap1 in *P. pastoris*.

In *P. pastoris*, only one non-peroxisomal glutathione peroxidase-encoding gene, *PpGpx1*, has been found, as in the case of *S. pombe* (ScGpx1), whereas three glutathione peroxidase homologs (ScGpx1, ScGpx2 and ScGpx3) have been found in *S. cerevisiae*. Also, we found the *P. pastoris* homolog of thioredoxin peroxidase, PpTsa1, from the genome sequence database.

To examine the possible existence of an upstream activator of PpYap1 under H$_2$O$_2$ stress, the sensitivities of the wild-type, *PpYAP1*, *PpGpx1*, and *PpTsa1*-deleted strains toward H$_2$O$_2$ were compared. The *Ppxpx1Δ* and *Pptsa1Δ* strains had decreased tolerance to H$_2$O$_2$, as was the case with the *Ppyap1Δ* strain (Fig. 3A). This phenotype, attributed to reduced peroxidase activity or a defective thioredoxin peroxidase, might have been caused by the absence of PpYap1 activation, or by both effects. Hence we determined the subcellular localization of mRed-PpYap1 in response to H$_2$O$_2$. In contrast to the wild-type and *Pptsa1Δ* strains, in the strain deleted for *PpGpx1*, mRed-PpYap1 failed to localize to the nucleus under these conditions (Fig. 3B). Furthermore, in the *Ppxpx1Δ* and *Ppgpx1Δ* strains, transcription of *Pptrri1* was not induced in response to H$_2$O$_2$ (Fig. 3C). In contrast, in the *Pptsa1Δ* strain, nuclear localization of mRed-PpYap1 and its transcriptional activation was not inhibited, but sensitivity towards tBOOH, the detoxification of which is dependent on activation of the PpYap1 pathway, was comparable to that of the wild-type strain (Fig. 3D). Hence we concluded that PpTsa1 is required for detoxification of H$_2$O$_2$ in a manner that does not involve the PpYap1 pathway, and that PpGpx1 is the transducer of the hydroperoxide signal to PpYap1 (see below for the mechanisms).

In *S. cerevisiae*, a H$_2$O$_2$ signal relay system was established, in which ScGpx3 induces ScYAP1, which in turn activates ScGpx2. On the one hand, this was based on the finding that the expression of the ScGpx3 gene was not induced by oxidative stress, whereas the induction of ScGpx1 and ScGpx2 by glucose starvation and oxidative stress respectively depended on ScYap1. On the other hand, the Scgpx3Δ strain was hypersensitive to hydroperoxides, whereas Scgpx1Δ and Scgpx2Δ did not show any obvious phenotypes.

In *S. pombe*, two oxidative stress responsive pathways are activated, one involving SpTpx1, which activates and is activated by Pap1. In the other pathway, the SpGpx1 gene is induced, and this induction is under the control of another bZIP transcription factor, Atf1, but not Pap1.

In the absence of *PpGpx1*, *P. pastoris* (**Ppgpx1Δ**) became very sensitive to H$_2$O$_2$ (Fig. 3A) and tBOOH (Fig. 3D), whereas transcription of *PpGpx1* was activated by H$_2$O$_2$ in a *PpYap1*-dependent manner (Fig. 2D). Therefore, *PpGpx1* is both an upstream activator and a target of *PpYAP1*, suggesting that it combines the functions of *ScGpx2* and *ScGpx3*. This H$_2$O$_2$ signal relay system (**Ppgpx1-PpYap1-PpGpx1 expression**) is not only distinct from that of *S. cerevisiae* (**ScGpx3-ScYap1-ScGpx2 expression**) but also from that of *S. pombe* (**SpTpx1-Pap1-SpTpx1 or SpAtf1-SpGpx1 expression**) since no *P. pastoris* homolog of *SpATF1* could be identified, and PpTsa1 (the homolog of SpTpx1) did not regulate the PpYap1 pathway during H$_2$O$_2$ stress (Fig. 3).
Fig. 3. PpGpx1 Is the H₂O₂ Signal Transducer of PpYap1.
A. Plate H₂O₂ sensitivity assay. The Ppgpx1Δ, Ppts1Δ, Ppyap1Δ, and wild-type strains were grown in SD medium and spotted on SD medium containing H₂O₂ (0.4 mM). Growth was inspected after 3 d at 28 °C. B. mRed-PpYap1 responses to H₂O₂ in a PpGpx1-dependent manner. The Ppgpx1Δ, Ppts1Δ, Ppyap1Δ, and wild-type strains expressing mRed-PpYap1 were grown in SD medium to the exponential phase and treated with H₂O₂ (0.4 mM, 10 min). C. The same strains as in (A) were used to measure the gene expression of PpTRR1 by qRT-PCR. Strains were grown in SD medium and treated with H₂O₂ (0.4 mM) for 1 h. The relative abundance of these mRNAs was standardized against the levels for PpACT1. Results are shown as means ± s.d. (n = 3). D. Plate tBOOH sensitivity assay. The same strains as in (A) were grown in SD medium and spotted on SD medium containing tBOOH (1.0 mM). Growth was inspected after 3 d at 28 °C.

Fig. 4. Identification of PpYap1 Redox-Active Cysteines.
A. PpYap1 cysteine residues important for tolerance to hydroperoxides. Wild type and Ppyap1Δ with vector and Ppyap1Δ expressing the wild type (Yap1), and mutant mRed-PpYap1 with the indicated cysteine residue or all (AAAAAA) cysteines replaced were tested for their tolerance to H₂O₂ (0.4 mM), tBOOH (1.0 mM), or CHP (25 μM) by spotting 10-fold serial dilutions on solid medium. Growth was inspected after 3 d at 28 °C. B. The same strains as in (A) were used to measure the gene expression of PpTRR1 by qRT-PCR, as in Fig. 3D. The cells were treated with 0.4 mM H₂O₂ for 1 h. The relative abundances of these mRNAs were standardized against the levels for PpACT1. Results are shown as means ± s.d. (n = 3).
Cysteine C373 and C414 are not required for the defense mechanism of PpYap1 against hydroperoxide stress

The Yap1 proteins form reversible, transient disulphide bonds between conserved cysteine residues in response to $H_2O_2$. Individual cysteine-to-alanine point mutations in four ScYap1 cysteine residues, Cys303, Cys310, Cys598, and Cys629, cause a defective response to $H_2O_2$. To determine whether the cysteine residues of PpYap1 CRD (Fig. 1) also play roles in regulating its function, we tested the capacity of cysteine substitution mutants to rescue the hypersensitivity of the Ppyap1/C1 strain to $H_2O_2$, tBOOH and CHP. PpYap1AAAAA, in which all cysteines in CRD were replaced by alanine, did not complement Ppyap1/C1. Analysis of individual cysteine-to-alanine substitution mutants showed that PpYap1C373A and PpYap1C414A conferred wild-type $H_2O_2$ tolerance (Fig. 4A), whereas all the other cysteine mutants (C234A, C240A, C392A, and C423A) failed to do so. PpYap1C234A slightly increased the tolerance to $H_2O_2$ and CHP as compared to the other three PpYap1 mutants.

The PpYap1 cysteine mutants were also tested for their capacity to activate PpTRR1 expression in response to $H_2O_2$ (Fig. 4B). Again, in this assay, Yap1C373A and Yap1C414A showed wild-type transcriptional activation. Furthermore, double cysteine mutants PpYap1C373A,C414A also showed a wild-type $H_2O_2$ tolerance and transcriptional activation (data not shown). In contrast, the other cysteine mutants (PpYap1C234A, PpYap1C240A, PpYap1C392A, and PpYap1C423A) did not activate PpTRR1 transcription upon $H_2O_2$ treatment. No significant increase in the level of transcriptional activation of the antioxidant genes tested was detected in strains expressing PpYap1C234A despite their relatively high tolerance to hydroperoxides.

Strains expressing the C234A, C240A, C392A, and C423A PpYap1 mutants had phenotypes similar to the Ppyap1Δ strain. All of these mutant strains were unable to grow or to activate PpTRR1 in the presence of $H_2O_2$. These results indicate that four cysteine residues (C234, C240, C392, and C423), which are highly conserved among Yap1 homologs (Fig. 1B), were all required for the PpYap1 response to hydroperoxides. The conserved cysteine residues of PpYap1, C234, C240, C392, and C423 corresponded to those of ScYap1, C303, C310, C598, and C629 respectively (Fig. 1).

Based on these conserved cysteine residues and the postulated $H_2O_2$-sensing mechanism of ScYap1, H$_2$O$_2$-sensing and -scavenging in P. pastoris perhaps occurs as follows: PpGpx1 is directly oxidized by $H_2O_2$ to yield H$_2$O and a sulfenic acid, PpGpx1-C36-SOH. The nascent C36-SOH reacts with PpYap1 C392 to form a PpGpx1-PpYap1 mixed disulphide. The PpGpx1-PpYap1 intermolecular disulphide linkage is then transposed to the intramolecular PpYap1 C240-S-S-C392 or C392-SS-C423 disulphide bridge, which is unstable and easily reduced, with recycling of reduced PpGpx1. After the formation of C240-S-S-C392, C234 and C423 form a second disulphide bond, resulting in stable activation of PpYap1. It appears that formation of C240-S-S-C392 or C392-SS-C423 disulphide bridges can support transient nuclear accumulation of PpYap1, and thus the strain expressing PpYap1C234A can show some increased tolerance. In contrast, PpYap1 C373 and C414 are not critical for the PpYap1 response to $H_2O_2$.

This scenario is supported by the following observations: (1) PpGpx1 has been found to be a signal transducer of $H_2O_2$ to PpYap1 (Fig. 3). (2) The limited growth by PpYap1C234A. This suggests that C234 is involved in the more stable intramolecular disulphide bonds of PpYap1.

The critical cysteine residues on PpYap1 CRD overlap between hydroperoxides defense and optimal growth on methanol

To assess the mechanism of PpYap1 response during growth on methanol medium (SM), the growth patterns...
of the wild-type. PpYap1Δ, and PpYap1 mutants were compared. All of the tested strains grew comparably well on SD medium (data not shown). As shown in Fig. 5, the PpYap1Δ strains expressing the PpYap1 and PpYap1 mutants C373A and C414A grew nearly like wild-type. The growth of the PpYap1C234A- and PpYap1C242A-mutants was retarded as compared to that of the wild-type strain. On the other hand, the strains expressing PpYap1C392A, PpYap1C423A, and PpYap1CAAATAA displayed severe growth defects, similar to that of the PpYap1Δ strain. These results indicate that as for the response to oxidative stress, C373 and C414 are not required for the function of PpYap1 during methanol metabolism, and that C234, C240, C392, and C423, which were required for tolerance to H₂O₂, are necessary for optimal growth on methanol. Furthermore, C392 and C423 in the c-CRD appear to play more important roles than the cysteines in n-CRD during growth on methanol (Fig. 5). This suggests that the nuclear export signal, which is present on c-CRD, is made inaccessible by mixed disulphides (or thiol adducts) on these cysteines, and that this is rate-limiting or sufficient during growth on methanol.

In summary, this paper describes the identification and molecular function of the ScYap1-homolog in the methylotrophic yeast P. pastoris. Our results indicate that PpYap1 plays significant roles not only in defense against various reactive agents, but also during physiological growth on methanol. The first step in methanol metabolism is the Aox-mediated oxidation of methanol, which not only produces H₂O₂ but simultaneously generates another toxic compound, formaldehyde. The glutathione-dependent oxidation pathway is considered to be involved in the detoxification of formaldehyde, and the genes involved in the glutathione redox system are targets of ScYap1. Recently we found evidence of regulation of the glutathione redox system by PpYap1 in P. pastoris during methanol metabolism, and we are currently trying to elucidate this further.

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