Yap1-Regulated Glutathione Redox System Curtails Accumulation of Formaldehyde and Reactive Oxygen Species in Methanol Metabolism of *Pichia pastoris*†

Taisuke Yano,1 Emiko Takigami,1 Hiroya Yurimoto,1 and Yasuyoshi Sakai1,2*

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan, and CREST, Japan Science and Technology Agency, 5, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan

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The glutathione redox system, including the glutathione biosynthesis and glutathione regeneration reaction, has been found to play a critical role in the yeast *Pichia pastoris* during growth on methanol, and this regulation was at least partly executed by the transcription factor PpYap1. During adaptation to methanol medium, PpYap1 transiently localized to the nucleus and activated the expression of the glutathione redox system and upregulated glutathione reductase 1 (Glr1). Glr1 activates the regeneration of the reduced form of glutathione (GSH). Depletion of Glr1 caused a severe growth defect on methanol and hypersensitivity to formaldehyde (HCHO), which could be complemented by addition of GSH to the medium. Disruption of the genes for the HCHO-oxidizing enzymes PpFld1 and PpFgh1 caused a comparable phenotype, but disruption of the downstream gene PpFDH1 did not, demonstrating the importance of maintaining intracellular GSH levels. Absence of the peroxisomal glutathione peroxidase Pmp20 also triggered nuclear localization of PpYap1, and although cells were not sensitive to HCHO, growth on methanol was again severely impaired due to oxidative stress. Thus, the PpYap1-regulated glutathione redox system has two important roles, i.e., HCHO metabolism and detoxification of reactive oxygen species.

Glutathione is a regulatory aspect of the glutathione redox ratio (GSH/GSSG), which is poorly understood (31).

Glutathione (L-γ-glutamyl-L-cysteinylglycine) assumes pivotal roles in bioreduction, protection against oxidative stress, detoxification of xenobiotics and endogenous toxic metabolites, transport, enzyme activity, and sulfur and nitrogen metabolism (28). Its biological significance comes from the free thiol group of the cysteine residue, which confers unique properties (30). In cells, glutathione mainly exists in the reduced form (GSH), as the oxidized form (GSSG) (9) is converted rapidly by Glr.

In yeast methanol metabolism, methanol is first oxidized to formaldehyde (HCHO) by a peroxisomal enzyme, alcohol oxidase. This reaction results in high levels of hydrogen peroxide (H$_2$O$_2$) (Fig. 1), which, like other reactive oxygen species (ROS) in methanol-induced peroxisomes, is scavenged by two peroxisomal antioxidant enzymes, Pmp20 (glutathione peroxidase) and catalase (1, 14, 15).

HCHO is an intermediate located at the branching point between the assimilation and dissimilation pathways (51). In the former, HCHO is fixed to xylulose 5-phosphate by dihydroxyacetone synthase within peroxisomes (33). Otherwise, HCHO nonenzymically reacts with GSH to form S-hydroxyethylglutathione, and subsequently, HCHO is oxidized to CO$_2$ via the dissimilation pathway (44, 51). S-Hydroxyethylglutathione formed in peroxisomes may be exported to the cytosol (14), where it acts as a substrate for GSH-dependent HCHO dehydrogenase. S-Formylglutathione produced by HCHO dehydrogenase is then hydrolyzed to GSH and formic acid by S-formylglutathione hydrolase. Finally, formic acid is oxidized to CO$_2$, by formate dehydrogenase.

Therefore, GSH is assumed to play critical roles during yeast methanol metabolism in two distinct functions, i.e., (i) protection against ROS and (ii) detoxification and oxidation of HCHO. Although these studies suggested a tight link between methanol metabolism and glutathione, the glutathione redox system, including its synthesis and recycling, especially in the regulatory aspect of the glutathione redox ratio (GSH/GSSG), is poorly understood (31).

In *Saccharomyces cerevisiae*, several genes of the glutathione redox system, such as the glutathione reductase gene (*GLR1*), the glutathione peroxidase gene (*GPX2*), and the gene that encodes the rate-limiting enzyme of the glutathione synthesis pathway (*GSH1*), are induced by the transcriptional regulator ScYap1, which responds to oxidative stress (8, 10, 11, 12, 16, 23, 35, 40, 48). ScYap1, a basic leucine zipper (bZIP) DNA binding protein of the AP-1 family, is activated upon exposure to peroxides or to thiol-modifying drugs such as diamide by a mechanism that acts at the level of subcellular localization (5, 6, 19, 20, 21, 22, 46, 50). ScYap1 is restricted to the cytosol, but upon exposure to ROS, Yap1 rapidly accumulates in the nucleus (21, 50). However, since cells were exposed to extracellularly supplemented oxidants in previous studies, the physiological role of Yap1 during normal cell growth has not been elucidated.

In this study, we pursued the regulatory mechanism of the glutathione redox system and its physiological significance dur-
Pichia pastoris. We examined the intracellular level of glutathione together with its redox ratio and analyzed the phenotypes of various strains in which one of the genes involved in the glutathione redox system was disrupted. Furthermore, we isolated and characterized the P. pastoris homologue of YAP1 (PpYAP1) and shed light on how the accumulation of HCHO and ROS is prevented by the glutathione redox system during yeast methanol metabolism.

MATERIALS AND METHODS

Strains and media. P. pastoris PPY12 (arg4 his4) (32) was used as the wild-type strain. Escherichia coli DH10B was routinely used for plasmid propagation. The P. pastoris strains were grown on either YPD medium (2% glucose, 1% Bacto yeast extract, 2% Bacto peptone) or YNB medium (0.67% yeast nitrogen base without amino acids) supplemented, when required, with an appropriate amino acid(s) (200 μg/ml for arginine, 200 μg/ml for histidine) or Zeocin (50 μg/ml; Invitrogen, Carlsbad, CA). The following were used as carbon sources in YNB medium: 2% (wt/vol) glucose (SD), 2% (vol/vol) methanol (SM), and 0.5% (vol/vol) oleate (SO). Tween 80 was added to the medium containing oleate at a concentration of 0.05% (vol/vol). We used 2% methanol to clearly detect the phenotypic differences between the wild type and gene disruptants, although similar differences could also be observed at lower methanol concentrations (e.g., 0.7%).

**FIG. 1.** The glutathione redox system and yeast methanol metabolism. Aox, alcohol oxidase; Das, dihydroxyacetone synthase; Cta, catalase; Fld, HCHO dehydrogenase; Fgh, S-formylglutathione hydrolase; Fdh, formate dehydrogenase; Pmp20, peroxisomal glutathione peroxidase; Gpx, glutathione peroxidase; GS-CH2OH, S-hydroxymethylglutathione; GS-CHO, S-formylglutathione; Xu5P, xylulose 5-phosphate; ROOH (where R is an aliphatic or aromatic organic group or simply hydrogen), alkyl hydroperoxide. Dashed arrow, hydroxyl radicals from H2O2 attack the peroxisomal membrane, resulting in the generation of ROOH (14). Yap1-targeted enzymes are boxed.

Expression of PpGlr1 and PpYap1 in P. pastoris. To visualize the localization of PpGlr1, a strain expressing a yellow fluorescent protein (YFP)-tagged PpGlr1 (PpGlr1-YFP) fusion protein was constructed as follows. First, a fragment with the 1-kb 5′ untranslated region and the coding region of PpGLR1 was obtained by PCR with primers PpGLR1-EcoRI-Fw and PpGLR1-SpeI-Rv (Table 1) by using genomic DNA as the template. Next, a 0.7-kb CiaI-NheI fragment from the downstream region of PpGLR1 was cloned into the wild-type strain by electroporation. After being linearized with PsI, pDyap1 was transformed into the wild-type strain by electroporation. The PCR products were recombined as NotI-PstI and PstI-XhoI fragments, respectively, with a 4.9-kb NotI-XhoI fragment of plasmid SK11001-Zeor which included the Zeocin resistance (Zeo r) gene (26), yielding the PpYAP1 disruption vector pDyap1. After being linearized with PstI, pDyap1 was transformed into the wild-type strain 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 with EcoRI-digested genomic DNA of transformants and a 0.7-kb CiaI-NaeI fragment from the downstream region of the PpYAP1 gene as the probe.

Expression of PpGlr1 and PpYap1 in P. pastoris. To visualize the localization of PpGlr1, a strain expressing a yellow fluorescent protein (YFP)-tagged PpGlr1 (PpGlr1-YFP) fusion protein was constructed as follows. First, a fragment with the 1-kb 5′ untranslated region and the coding region of PpGLR1 was obtained by PCR with primers PpGLR1-EcoRI-Fw and PpGLR1-SpeI-Rv (Table 1) by using genomic DNA as the template. Next, YFP was amplified by PCR with primers
and ReverTra Ace (Toyobo). After reverse transcription for 50 min at 42°C, RNase H was added. qRT-PCR was performed in 20-repetitions, 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s (all temperature performed with 1 μl mixtures in glass capillaries tubes in a LightCycler (Roche Diagnostic). The PCR cycling reaction was with an RNeasy mini kit (Qiagen) monitored by on-column DNase digestion. The amounts of mRNAs was standardized against the levels for PpACT1 (29), was 70 to 60% lower than that in glucose- or oleate-grown cells as a single carbon source. The total amount of glutathione was more than twofold higher in cells grown on methanol (SM) than in cells grown on glucose (SD) or oleate (SO) (Fig. 2A). In methanol-grown cells, the intracellular GSH/GSSG ratio, the best index of the cellular thiol redox balance (29), was 70 to 60% lower than that in glucose- or oleate-grown cells (Fig. 2B), indicating that the intracellular redox state of thios in methanol-grown cells is more toward their oxidized form. Since oleate metabolism did not alter the glutathione redox state by more than 15%, the intracellular redox regulation observed during methanol metabolism does not seem to be a general feature of peroxisome metabolism but appears specific to methanol metabolism. Therefore, glutathione metabolism during growth on methanol was studied further.

To visualize the localization of PpYap1, a strain expressing a DsRed-monomer fluorescent protein (mRed)-PpYap1 fusion protein was constructed as follows. To investigate the role of glutathione in relation to peroxisome metabolism, the total amounts of glutathione (GSH plus GSSG) were compared among cells grown on methanol, oleate, or glucose as a single carbon source. The total amount of glutathione was more than twofold higher in cells grown on methanol (SM) than in cells grown on glucose (SD) or oleate (SO) (Fig. 2A). In methanol-grown cells, the intracellular GSH/GSSG ratio, the best index of the cellular thiol redox balance (29), was 70 to 60% lower than that in glucose- or oleate-grown cells (Fig. 2B), indicating that the intracellular redox state of thios in methanol-grown cells is more toward their oxidized form. Since oleate metabolism did not alter the glutathione redox state by more than 15%, the intracellular redox regulation observed during methanol metabolism does not seem to be a general feature of peroxisome metabolism but appears specific to methanol metabolism. Therefore, glutathione metabolism during growth on methanol was studied further.

Adaptation to methanol culture elevated the total amount of glutathione and decreased the intracellular GSH/GSSG ratio. From the P. pastoris genome sequence database, we identified the PpGLR1, PpGPX1, and PpFDH1 genes by homology searches. While three glutathione peroxidase genes (ScGPX1, ScGPX2, and ScGPX3) were found in S. cerevisiae (16), only one was found in P. pastoris (PpGPX1), as in Schizosaccharomyces pombe (SpGPX1) (49). Next, we examined the transcriptional levels of PpGLR1, PpGPX1, and PpGSGL1 during growth on methanol by qRT-PCR analysis. Among them, PpGPX1 was found to be the most highly induced during growth on methanol (Fig. 2C).

Growth properties of gene disruptants on methanol. We hypothesized that mutants with the glutathione redox system deleted would show a growth defect on methanol. It has been reported that GSH-deficient mutants of the methylotrophic yeast Hansenula polymorpha failed to grow on methanol due to the accumulation of toxic amounts of HCHO (37).

To investigate the role of the glutathione redox system and its putative regulator PpYap1, the growth on methanol of PpGLR1, PpGPX1, PpPMP20, and PpYAP1 mutant strains was compared. The PpYAP1Δ mutant strain completely lost the
Gene that encodes Glr in the supplemental material), indicating that there is only one VOL. 8, 2009 ROLES OF PpYap1-REGULATED GLUTATHIONE REDOX SYSTEM 543 activity was detected in the Pp cells did. Especially in the Pp disruptants tested showed lower GSH/GSSG ratios than wild-type (Fig. 3B and C). During growth on methanol, all of the disruptants showed lower GSH/GSSG ratios when they were grown on methanol compared to those of wild-type cells, with the glutathione peroxidase activity of PpPmp20 requires the glutathione peroxidase activity of PpPmp20 requires catalyzed regeneration of GSH has a critical role in HCHO detoxification. Indeed, PpPmp20Δ mutant cells in mid-exponential phase on methanol were diluted to fresh methanol medium, the lag phase was shorter for the second culture (data not shown), suggesting a higher level of GSH at the exponential phase of methanol-glucose-grown PpGlr1Δ mutant cells. We tested whether the growth defect of the PpGlr1Δ mutant cells on methanol could be complemented by adding GSH or GSSG to the culture medium (Fig. 3D, left panel). The retarded growth of the PpGlr1Δ mutant was partially complemented by the presence of GSH (50 µg/ml) but not by the presence of GSSG (50 µg/ml). In the presence of GSH (200 µg/ml), the growth of the PpGlr1Δ mutant strain was comparable to that of wild-type cells (data not shown). On the other hand, addition of GSH (50 µg/ml) did not affect its growth on glucose (Fig. 3D, right panel). These results indicate that GSH, not GSSG, plays a critical role during methanol metabolism in methylotrophic microorganisms.

Function of GSH during methanol metabolism. We next asked whether the growth defect of the gene disruptants mainly came from the failure to detoxify either HCHO or ROS. First, the HCHO levels in the culture medium of the gene disruptants were determined (Fig. 4A). In the wild-type strain, HCHO accumulation peaked after 20 to 30 h of incubation and then gradually decreased. The maximum HCHO levels in the Ppgpr1Δ, Pppyp1Δ, and Ppppmp20Δ mutant strains were twofold higher than in wild-type cells, with the highest peak level in the Ppgpr1Δ mutant. Disruption of PpPMP20, which encodes a glutathione peroxidase specifically involved in the detoxification of ROS but not in that of HCHO, resulted in an almost constant high level of HCHO. As shown in Fig. 4B, this high HCHO level is owing to the remarkably small PpPmp20Δ mutant cell numbers, since this strain was inviable on methanol due to ROS generated through methanol metabolism. In contrast, the Ppgpr1Δ mutant strain, which also had a severe growth defect on methanol, was hypersensitive to HCHO, suggesting that GSH, which has been found to function mainly in the detoxification of HCHO during growth on methanol (51), is not sufficiently abundant to sustain viability under these growth conditions. This is in line with the high HCHO levels (Fig. 4A) and low GSH concentrations (Fig. 3C) detected in this strain.

Intracellular localization of the glutathione regeneration system in P. pastoris. Our present data indicate that Glr1-catalyzed generation of GSH has a critical role in HCHO detoxification during methanol metabolism. On the other hand, the glutathione peroxidase activity of PpPmp20 requires GSH within peroxisomes for the detoxification of ROS (14). In other yeasts, such as S. cerevisiae and S. pombe, glutathione reductase showed a bimodal distribution between mitochondria and the cytosol (27,39). In addition, this enzyme was reported to be localized in peroxisomes of pea (Pisum sativum) leaves (7,18). Therefore, we examined the localization of

FIG. 2. Effects of carbon sources on the glutathione system. Wild-type P. pastoris was grown on methanol (SM), glucose (SD), or oleate (SO) medium to the mid-exponential phase. (A) Intracellular levels of total glutathione (GSH plus GSSG). (B) Intracellular glutathione redox ratio (GSH/GSSG). (C) Relative transcriptional levels of genes involved in the glutathione redox system on methanol compared to those on glucose. The relative abundance of these mRNAs was standardized against the levels for PpACT1. Results are means ± standard deviations (n = 3).
PpGlr1 during the methylotrophic growth of *P. pastoris*. We expressed PpGlr1 C-terminally tagged with YFP (PpGlr1-YFP) under the control of its native promoter (*PGLR1*) in the *Pp*glr1/H9004 mutant strain and compared the fluorescence to that obtained for a CFP-tagged peroxisomal marker, SKL (25). PpGlr1-YFP localized to the mitochondria and the cytosol during growth on methanol (Fig. 5A), glucose, or oleate (data not shown). The localization of PpGlr1 was also analyzed in the wild-type strain by subcellular fractionation, which separated the intracellular components into a cytosolic supernatant and an organelle pellet consisting mainly of peroxisomes and mitochondria. The glutathione reductase activity colocalized with the mitochondrial marker protein cytochrome c oxidase (4) on a Histodenz gradient (Fig. 5B) but not with peroxisomal catalase. Previously, we showed that methanol-induced peroxisomes purified from the methylotrophic yeast *Candida boidinii* also did not exhibit any detectable glutathione reductase activity (14). Therefore, we found no evidence of the presence of a glutathione regeneration system within peroxisomes of methylotrophic yeasts.

FIG. 3. Phenotypes of strains carrying disruptions of genes involved in the glutathione redox system. (A) Growth properties of the wild type (WT) and the *Pp*glr1Δ, *Pp*gpx1Δ, *Pp*pmp20Δ, and *Pp*yap1Δ disruptants on methanol (SM). The intracellular total glutathione levels (B) and the intracellular GSH/GSSG ratios (C) in the wild type and the *Pp*glr1Δ, *Pp*gpx1Δ, *Pp*pmp20Δ, and *Pp*yap1Δ disruptants grown on methanol (SM), glucose (SD), or oleate (SO) medium are also shown. Results are means ± standard deviations (*n* = 3). (D) Growth of the wild type and the *Pp*glr1Δ disruptant on minimal methanol (SM, left panel) or glucose (SD, right panel) medium not supplemented or supplemented with 50 μg/ml GSH or GSSG.
PpYap1 transiently localized to the nucleus and positively regulated the expression of genes related to the glutathione redox system during adaptation to methanol. In *S. cerevisiae*, the nuclear enrichment of transcription factor ScYap1 in response to oxidative stress (21, 50) resulted in the transcriptional activation of genes that encode enzymes related to the glutathione redox system, such as ScGLR1, ScGPX2 (one of the three glutathione peroxidase genes), and ScGSH1, as well as numerous genes that are involved in preventing oxidative damage to the cells (17, 19, 35, 40, 43). To investigate the role and behavior of Yap1 in *P. pastoris*, we have cloned the PpYAP1 gene.

We expressed a DsRed-monomer–PpYap1 fusion protein (mRed-PpYap1) under the control of the native promoter (PYAP1) in the Ppyap1Δ mutant strain and confirmed this by Western blot analysis (data not shown). The Ppyap1Δ mutant strain was hypersensitive to various oxidative stresses, and under oxidative stress conditions, mRed-PpYap1 accumulated in the nucleus (T. Yano and Y. Sakai, unpublished results). These experiments confirmed that activation of mRed-PpYap1 can be monitored in real time by its accumulation in the nucleus, as observed in *S. cerevisiae*.

The Ppyap1Δ mutant strain also exhibited a prolonged lag phase (Fig. 3A) and accumulated a considerable amount of HCHO (Fig. 4A), suggesting that PpYap1 is involved in the detoxification of HCHO during growth on methanol. To see the activation of PpYap1 under physiological conditions, the mRed-PpYap1-expressing Ppyap1Δ mutant cells were grown to exponential phase in glucose medium (SD) and then shifted to methanol or oleate medium. mRed-PpYap1 was predominantly localized to the cytosol before the shift. At 3 to 14 h after a shift to methanol medium, mRed-PpYap1 was transiently localized to the nucleus (Fig. 6A and B). In contrast, mRed-PpYap1 stayed in the cytosol during growth on glucose or oleate (data not shown).

This transient nuclear localization of PpYap1 in methanol-grown cells appears to activate the expression of genes related to the glutathione redox system (PpGLR1, PpGPX1, and PpGSH1); as in the Ppyap1Δ mutant, the transcriptional activation of these genes was suppressed (Fig. 6C). The promoters of these genes contain various putative Yap1 response elements (13; data not shown). These results are consistent with the finding that the total glutathione amount and the GSH/GSSG ratio were lower in the Ppyap1Δ mutant strain during growth on methanol than in the wild-type strain (Fig. 3B and C). The retarded growth of the Ppyap1Δ mutant strain on methanol and HCHO accumulation may be due to the retarded expression of these genes, especially *PpGLR1*.

**Activation of PpYap1 in various mutants.** Since we identified a novel physiological role for PpYap1 in HCHO detoxification, we studied how PpYap1 is activated during adaptation to methanol in various gene disruptants. mRed-PpYap1-expressing strains were precultured in glucose medium (SD) and shifted to methanol medium (SM containing 0.05% yeast ex-
In the wild-type strain when these strains were grown on methanol, PpYap1 accumulated in the nucleus at earlier time points than mRed-PpYap1 expressing cells incubated on methanol for 6 h were stained with DAPI. Bars, 2 μm. (A) Subcellular localization of mRed-PpYap1. The PpYap1Δ mutant strain expressing mRed-PpYap1 was grown in glucose (SD) medium to exponential phase and shifted to methanol (SM) medium. (B) Nuclear accumulation of mRed-PpYap1. (C) Relative transcriptional levels of PpGLR1, PpGPX1, and PpGSH1 in the Ppyap1Δ mutant compared to those in the wild type. The relative abundance of these mRNAs was standardized against the levels for PpACT1. Results are means ± standard deviations (n = 3).

FIG. 6. mRed-PpYap1 is transiently localized to the nucleus and positively regulates the transcription of genes related to the glutathione redox system during methanol metabolism. (A) Subcellular localization of mRed-PpYap1. The PpYap1Δ mutant strain expressing mRed-PpYap1 was grown in glucose (SD) medium to exponential phase and shifted to methanol (SM) medium. (B) Nuclear accumulation of mRed-PpYap1 during growth on methanol. mRed-PpYap1-expressing cells incubated on methanol for 6 h were stained with DAPI. Bars, 2 μm. (C) Relative transcriptional levels of PpGLR1, PpGPX1, and PpGSH1 in the PpYap1Δ mutant compared to those in the wild type. The relative abundance of these mRNAs was standardized against the levels for PpACT1. Results are means ± standard deviations (n = 3).

to support the growth of the mutants) to monitor the localization of mRed-PpYap1 periodically (time, 1 to 24 h).

First, we tested mutations affecting the GSH-dependent HCHO oxidation pathway (Ppfdh1Δ, Ppgfh1Δ, and PpYap1Δ). In cells with these mutations, the intracellular HCHO level was expected to increase without enhancing the generation of ROS (Fig. 1), which made the Ppfdh1Δ and Ppgfh1Δ mutants (but not the PpYap1Δ mutant) hypersensitive to HCHO in the medium (see Fig. S2B in the supplemental material). As shown in Fig. 7, in both the Ppfdh1Δ and Ppgfh1Δ mutant strains, mRed-PpYap1 accumulated in the nucleus at earlier time points than in the wild-type strain when these strains were grown on methanol. This nuclear PpYap1 localization could not be released in Ppfdh1Δ and Ppgfh1Δ mutant cells growing on glucose medium to which HCHO was added (see Fig. S2A in the supplemental material). Moreover, both the Ppfdh1Δ and Ppgfh1Δ mutant strains showed HCHO sensitivity but the PpYap1Δ mutant strain did not (see Fig. S2B in the supplemental material). These results indicate that HCHO toxicity or the deficiency of GSH triggers the nuclear localization of PpYap1 in the PpYap1Δ and Ppgfh1Δ mutant strains and suggested activation of PpYap1 by HCHO. We also confirmed that HCHO itself could induce the nuclear accumulation of mRed-PpYap1 (Yano and Sakai, unpublished).

P. pastoris has two peroxisomal antioxidant enzymes, i.e., peroxisomal catalase Cta1 and Pmp20 glutathione peroxidase; a homologue of cytosolic catalase CTT1 could not be found in the P. pastoris genome. The growth defect of the PpPmp20Δ mutant was more severe on methanol medium than that of the PpCta1Δ mutant (Fig. 4B; see Fig. S2C in the supplemental material), as was the case for their C. boidinii homologues (15). In the PpPmp20Δ mutant strain, mRed-PpYap1 localized to the nucleus 1 to 2 h after a shift to methanol medium, whereas this took another hour in PpCta1Δ mutant and wild-type cells (Fig. 7). Thus, increased levels of peroxisomal ROS resulting from the lack of PpPmp20 activity cause a more rapid activation of PpYap1 than H2O2 accumulation, which could only be detected in the absence of PpCta1 (data not shown). HCHO by itself did not cause a growth defect in the PpPmp20Δ mutant, but methanol as a carbon source did (Fig. 4B). It is the conversion of methanol to HCHO that causes the generation of ROS as a by-product (Fig. 1), which is thus most damaging to PpPmp20Δ mutant cells. Taken together, our results show that the absence of PpPmp20 made the cells highly susceptible to oxidative stress.

**DISCUSSION**

Previously, a GSH biosynthetic mutant was reported to be impaired in growth on methanol (31). However, with such a mutant it has been difficult to show that GSH has two distinct physiological roles, i.e., (i) elimination of ROS and (ii) detoxification of HCHO, because the mutant simultaneously lost both functions. Our analyses of various mutations involved in GSH-related metabolism show that GSH indeed has these two distinct roles and have revealed a novel role for yeast Yap1, which is normally associated with oxidative stress, under physiological condition in HCHO detoxification.

We detected a significant level of HCHO in the culture medium of methanol-grown *P. pastoris*, which increased in all of the mutants tested. In the Ppgfh1Δ mutant strain, a mutant deficient in GSH regeneration from GSSG, the highest level of HCHO was detected at an early stage of growth in methanol (Fig. 4A). Although GSH nonenzymatically reacts with HCHO in vitro (51), this and other experiments confirm in vivo that GSH, not GSSG, functions in HCHO detoxification and that both the presence of GSH and its Glr1-catalyzed regeneration from GSSG are essential for methanol metabolism in yeast.

Pmp20, a peroxisomal glutathione peroxidase, rather than catalase, has been shown to be the main scavenger of ROS during methanol metabolism by *P. pastoris*. In vivo, methanol is converted in a peroxisomal alcohol oxidase-catalyzed reaction...
to HCHO and H$_2$O$_2$ (1). Subsequently, H$_2$O$_2$ causes the formation of ROS. As the Pp$_{pmp20}$/H9004 mutant strain was sensitive to methanol but not to HCHO (Fig. 4B), the growth defect of the Pp$_{pmp20}$/H9004 mutant is due to the failure to detoxify ROS. On the other hand, the Pp$_{glr1}$/H9004 mutant strain was not only sensitive to methanol but also very sensitive to HCHO (Fig. 4B), indicating the importance of the regeneration of GSH from GSSG. Therefore, in addition to ROS elimination, GSH has a significant role in the detoxification of methanol-derived HCHO.

We determined and compared the redox ratio of glutathione among various cells. The intracellular GSH/GSSG ratio becomes more oxidized in methanol-grown cells than in oleate-or glucose-grown cells. Moreover, the total glutathione level was higher in methanol-grown cells (Fig. 2A). Since much of GSH is assumed to be trapped by HCHO (Fig. 1) for further metabolism, the shifted intracellular redox state of methanol-grown cells may result in the upregulation of de novo GSH synthesis. The Ppglr1Δ mutant strain exhibited a remarkably low GSH/GSSG ratio due to the block in the GSH regeneration reaction, but its total glutathione level was enhanced, indicating that de novo GSH synthesis supported the slow growth (Fig. 3). These results show that the regulation of the glutathione redox system plays important roles during adaptation to methanol.

PpYap1-dependent activation of the glutathione redox system, e.g., PpGlr1, PpGpx1, and PpGsh1, was found to occur during methanol adaptation and also in the Ppglr1Δ mutant strain. Indeed the Ppyap1Δ mutant strain exhibited a prolonged lag phase and accumulated a considerable amount of HCHO (Fig. 3A and 4A). Distinct from PpGpx1, expression of PpGLR1 and PpGSH1 was not completely abolished in the Ppyap1Δ mutant strain. Activation of the latter two genes might be mediated by some other factors, e.g., the Met4 homolog in P. pastoris (47).

Activation of PpYap1 in P. pastoris can be monitored by its localization to the nucleus (Yano and Sakai, unpublished). We observed that mRed-PpYap1 transiently localized to the nucleus after 4 to 12 h of methanol adaptation (Fig. 6A). Interestingly, the nuclear localization of mRed-PpYap1 was observed at earlier time points in HCHO oxidation pathway mutants, the Pp$_{fld1}$Δ mutant (after 1 h of adaptation) and the Pp$_{fgh1}$Δ mutant (after 3 h of adaptation). Moreover, in both of these mutant strains, HCHO accumulated to a considerably higher level than in the wild-type strain (see Fig. S2D in the supplemental material), with the HCHO level in the Pp$_{fld1}$Δ mutant being higher than that in the Pp$_{fgh1}$Δ mutant. Because both enzymes have not been shown to be involved in ROS elimination, their disruption stalls the regeneration of GSH as the intermediate S-hydroxymethylglutathione or S-formylglutathione cannot be processed (Fig. 1). Thus, either depletion of GSH or accumulation of HCHO is toxic for the cells and could trigger the activation of PpYap1 in these gene disruptants.

Cta1 and Pmp20 are the major peroxisomal antioxidant enzymes. Our present and preceding studies revealed that pmp20Δ mutant strains exhibited more severe phenotypes than cta1Δ mutant strains (15; see Fig. S2C in the supplemental material). PpYap1 activation, as indicated by its nuclear accumulation, which occurred at an earlier time point, suggesting that oxidative stress caused by ROS that could not be removed by Pmp20 activity, was more severe than that imposed by H$_2$O$_2$, accumulating in the absence of catalase activity. In other yeasts, Yap proteins have been identified as transcription fac-

![FIG. 7. Subcellular localization of mRed-PpYap1 in various mutants on methanol. Mutants carrying mRed-PpYap1 were grown in glucose (SD) medium to exponential phase and then shifted to methanol (SM) medium containing yeast extract (0.05%). Bar, 2 μm. WT, wild type.](image-url)
The intracellular redox state maintained by glutathione metabolism has been found to be finely regulated during the growth of *P. pastoris* on methanol. GSH/GSSG redox becomes more oxidized in cells growing in methanol than in cells growing in glucose or oleate. PpYap1 transiently localizes to the nucleus and activates the expression of PpGLR1 and other target genes, and upregulated PpGLR1 regenerates GSH (Fig. 8). Therefore, the glutathione redox system has critical physiological roles during growth on methanol, i.e., detoxification and oxidation of HCHO and elimination of ROS. Yeast methanol metabolism provides a good model system to study the physiological role and regulation of glutathione metabolism together with its redox homeostasis.

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