

Msn5p Is Involved in Formaldehyde Resistance but Not in Oxidative Stress Response in the Methylophilic Yeast *Candida boidinii*

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Methylophilic yeasts, which can utilize methanol as sole carbon and energy source, are exposed to two toxic metabolic intermediates, formaldehyde and hydrogen peroxide, during growth on methanol. Here we report that Msn5p, an importin- β family nuclear exporter, participated in the formaldehyde resistance mechanism but not in the hydrogen peroxide resistance mechanism in *Candida boidinii*. Disruption of the *MSN5* gene in this yeast caused retardation of growth on formaldehyde-generating growth substrates such as methanol and methylamine, but the expression levels of the methanol-metabolizing enzymes did not fall. The Msn5p-depleted strain was sensitive to formaldehyde but not to hydrogen peroxide. Furthermore, a yellow fluorescent protein-tagged Msn5p was diffuse in the cytoplasm of *C. boidinii* when the cells were treated with high concentrations of formaldehyde or ethanol, but was predominantly associated with the nuclei following treatment with hydrogen peroxide.

Key words: formaldehyde; hydrogen peroxide; *MSN5*; *Candida boidinii*; *Pichia pastoris*

Formaldehyde (HCHO) and hydrogen peroxide (H_2O_2) are both highly reactive compounds that have toxic effects on all organisms. Hence all organisms have cellular functions to cope with and detoxify these compounds. In the methanol metabolism of methylophilic yeasts, which are capable of utilizing methanol as sole carbon and energy source, HCHO and H_2O_2 are initially generated by oxidation of methanol.^{1–3)} Methylophilic yeasts, such as *Pichia pastoris*, *Hansenula polymorpha*, and *Candida boidinii*, are widely used in the production of heterologous proteins.^{4–8)} Because accumulations of HCHO and H_2O_2 can affect cell growth and protein productivity, it is necessary to elucidate cellular strategies to minimize the toxicity of these compounds.

In methylophilic yeasts, methanol is first oxidized to HCHO by alcohol oxidase (AOD) within peroxisomes, resulting in the formation of H_2O_2 , which is decomposed by peroxisomal catalase.¹⁾ A peroxiredoxin,

peroxisomal glutathione peroxidase (Pmp20), is responsible for the elimination of alkyl hydroperoxide species derived from H_2O_2 .⁹⁾ HCHO is a central intermediate, located at the branch point between the assimilation and the dissimilation pathway. In the assimilation pathway, fixation of HCHO with xylulose-5-phosphate into dihydroxyacetone and glyceraldehyde-3-phosphate is catalyzed by dihydroxyacetone synthase within the peroxisomes. On the other hand, HCHO is oxidized to CO_2 by a glutathione (GSH)-dependent HCHO oxidation pathway in the cytosol. In this pathway, HCHO reacts non-enzymatically with a reduced form of GSH to generate S-hydroxymethyl glutathione (S-HMG). Thereafter, S-HMG is used as a substrate for GSH-dependent formaldehyde dehydrogenase (FLD) to yield S-formylglutathione (S-FG). S-FG is then hydrolyzed to formate and GSH by S-formylglutathione hydrolase. Finally, formate is oxidized to CO_2 by formate dehydrogenase. We have elucidated the physiological importance of the enzymes involved in the detoxification of HCHO and H_2O_2 by cloning and disrupting the corresponding genes in *C. boidinii*.^{9–14)} Furthermore, we found recently that the GSH redox system, including GSH biosynthesis and regeneration reactions, plays critical roles in yeast methanol metabolism, viz., detoxification and oxidation of HCHO and protection against reactive oxygen species (ROS) derived from H_2O_2 . In addition, a transcription factor termed Yap1p plays an important role in the upregulation of GSH-related enzymes in *P. pastoris*.^{15,16)} In that the PpYap1p-depleted strain proved sensitive to H_2O_2 but not to HCHO, methylophilic yeasts must have an alternative transcriptional regulatory system to control the expression of the genes for HCHO resistance.

To date, in the budding yeast *Saccharomyces cerevisiae*, stress-inducible transcription factors Msn2p/Msn4p and heat shock protein Ssa4p, and their carrier Msn5p, have been reported to play important roles in protecting cells from stress-induced cell death.^{17–21)} Among these factors, we focused on Msn5p, which belongs to the importin- β family and participates in the nuclear export of several proteins. Msn5p is not only

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Abbreviations: AOD, alcohol oxidase; Msn5p-YFP, CbMsn5p tagged with YFP at the C-terminus; DAPI, 4',6'-diamidino-2-phenylindole; FLD, formaldehyde dehydrogenase; GSH, glutathione; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; RPA, replication protein A; S-FG, S-formylglutathione; S-HMG, S-hydroxymethyl glutathione; Zeo^r, zeocin-resistant

Table 1. Oligonucleotide Primers Used in This Study

Primer name	5'-sequence-3'
PpMSN5-up-PstI	AACTGCAGATTGTGTGAAGACATTTTG
PpMSN5-up-KpnI	GGGGTACCAGTGGATCCCAGATATTCTC
PpMSN5-down-SacII	TCCCCGCGGGCAGATAACGGATGGAATAT
PpMSN5-down-PstI	AACTGCAGGAAGAGGAACCTTCTAACAG
CbMSN5-up-EcoRI	CGGAATTCCCATCACCTTCTCTCAAGCAATCAC
CbMSN5-up-XhoI	CCGCTCGAGGTAGCCAATTCGACAACCCATTGTCTAA
CbMSN5-down-SacII	TCCCCGCGGGTAGACCCTGAATTATCAGCTTGTC
CbMSN5-down-EcoRI	CGGAATTCCTTCGAGAAGATGACGACGACTAATATAGC
SacI-CbMSN5-Fw	CCGAGCTCATAGCTGTCTGTGGCTCGAGGT
SbfI-CbMSN5-Rv	AAACGGCCTGCAGGTTTTTTTTTAAAGATGCAATGAATTGTAAAAATGCATTTCTCTTTG
PstI-YFP-Fw	AACTGCAGGTTTCTAAAGGTGAAGAATTATTC
NheI-YFP-Rv	CTAGCTAGCTTATTTATATAATTCATCCATACC
PpACT1-RT-Fw	GCCGGTAGAGATTGACCGACTACTTGATG
PpACT1-RT-Rv	GTAAGTGGTTTGGTCGATACCAGAAGCCTC
PpAOX1-RT-Fw	CCAAAGAAGCCAAGTCACAAGATCTACCGT
PpAOX1-RT-Rv	ACACCAGCTTCGATACCGTTAGTGGAAGA
PpFLD1-RT-Fw	TGAGTGCAGAGAGTGCAAGTTCTGTCTGTC
PpFLD1-RT-Rv	CCAATACATCCAGCACCAACACACCGATC

The underlined nucleotide sequences are additional restriction enzyme recognition sequences.

involved in nuclear export of the proteins that function in regulating carbohydrate metabolism, cell stress response, signal transduction control, cell cycle control, and so on, but also in the nuclear import of replication protein A (RPA), which is involved in DNA replication, DNA repair, and recombination.²²⁾ Disruption of *ScMSN5* caused lower survival rates than that of wild-type cells when cells were exposed to heat, starvation, ethanol, and oxidative stress conditions.²¹⁾ Thus Msn5p is required for responses to various kinds of stress.

Here we studied the physiological function of Msn5p in the methylotrophic yeast by gene disruption analysis and observation of the subcellular localization of Msn5p. Our results provide insight into the defense mechanism against HCHO.

Materials and Methods

Strains, media, and cultivation conditions. Haploid strain *C. boidinii* S2 was used as the wild-type strain.²³⁾ *C. boidinii* strain TK62 (*ura3*) was used as host for transformation.²⁴⁾ *P. pastoris* PPY12 (*arg4 his4*) was used as the wild-type strain.²⁵⁾ *Escherichia coli* DH10B (Takara Bio, Kyoto, Japan) and SA116²⁴⁾ were used for plasmid propagation.

The yeast strains were grown on either YPD medium (2% glucose, 2% Bacto peptone, and 1% Bacto yeast extract) or YNB medium (0.67% Yeast Nitrogen Base without amino acids), supplemented, when required, with appropriate amino acids (200 µg/mL for arginine and 200 µg/mL for histidine) or Zeocin (Invitrogen, Carlsbad, CA) (50 µg/mL or 100 µg/mL). One or more of the following were used as carbon sources in YNB medium: 2% (wt/vol) glucose, 2% (vol/vol) glycerol, 1% (vol/vol) methanol, 1% (vol/vol) ethanol, 0.5% (wt/vol) methylamine, and 0.5% (wt/vol) choline. In plate assays, the medium was supplemented with 1 mM H₂O₂, 2 mM HCHO, or 9 mM HCHO. The initial pH of the medium was adjusted to 6.0. Cultivation was performed at 28 °C under aerobic conditions with reciprocal shaking, and the growth of the yeast was monitored by measuring the optical density at 610 nm. HCHO was monitored by the method of Nash.²⁶⁾

E. coli was grown at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl), supplemented, when required, with ampicillin (50 µg/mL) or zeocin (50 µg/mL).

Construction of CbMSN5 and PpMSN5 deletion strains. A deletion cassette for the *CbMSN5* gene was constructed as follows: Primer pairs CbMSN5-up-EcoRI/CbMSN5-up-XhoI and CbMSN5-down-SacII/CbMSN5-down-EcoRI (Table 1) were used to amplify 1.0-kb and 1.1-kb fragments using genomic DNA as template. Three fragments,

the 1.0-kb EcoRI-XhoI fragment, the 1.1-kb SacII-EcoRI fragment, and the 4.1-kb XhoI-SacII fragment of plasmid SK⁺-*Zeo*^r (for *C. boidinii*²⁷⁾) harboring *Zeo*^r gene, were ligated, yielding *CbMSN5* disruption vector pDCbmsn5. pDCbmsn5 was digested with EcoRI, and then the 6.2-kb fragment was introduced into *C. boidinii* TK62 using a modified version of the lithium acetate method.²⁴⁾ Zeocin-resistant colonies were selected on YPD medium supplemented with zeocin. Disruption of the *CbMSN5* gene was confirmed by Southern blot analysis using BglII digested genomic DNA from the transformants and a 1.0-kb EcoRI-XhoI PCR fragment from the coding region of the *CbMSN5* gene as probe. The *Cbmsn5Δ* strain was converted to uracil prototrophy by transforming it with plasmid pNOTeI, which contains the *CbURA3* coding region.²⁸⁾ The nucleotide sequence of *CbMSN5* was deposited in DDBJ under accession no. AB669026.

In a similar way, a deletion cassette for the *PpMSN5* gene (PAS.chr4.0654) was constructed as follows: Two fragments of the *PpMSN5* gene (0.98-kb and 0.97-kb) were amplified by PCR with primer pairs PpMSN5-up-PstI/PpMSN5-up-KpnI and PpMSN5-down-SacII/PpMSN5-down-PstI (Table 1) respectively with genomic DNA as template. The PCR products were recombined as PstI-KpnI and SacII-PstI fragments respectively and ligated with the 4.9-kb KpnI-SacII fragment of plasmid SK⁺-*Zeo*^r, which includes the zeocin-resistant (*Zeo*^r) gene, yielding *PpMSN5* disruption cassette pDmsn5. After linearization with PstI, pDPpmsn5 was used to transform the wild-type strain by electroporation. Zeocin-resistant colonies were selected on YPD medium supplemented with zeocin. Disruption of the *PpMSN5* gene was confirmed by Southern blot using ClaI-XbaI-digested genomic DNA from the transformants and a 0.98-kb PstI-KpnI PCR fragment from the coding region of the *PpMSN5* gene as probe.

Construction of strains expressing the ScPHO5 gene under the control of various promoters. Plasmids pAPU²⁹⁾ and pFLPU (Yurimoto and Sakai, unpublished results), harboring the *S. cerevisiae* *PHO5* gene, encoding acid phosphatase, under the control of the gene promoters of *C. boidinii* *AOD1* and *FLD1* respectively, were introduced into the *Cbmsn5Δura3* strain. Integration of the plasmids into the genomes of the transformants was analyzed by Southern blotting of EcoRI-digested chromosomal DNA with the BamHI-PstI fragment of the *URA3* gene as probe (data not shown). Transformants that showed a single integration event of pAPU or pFLPU at the *ura3* locus in *Cbmsn5Δ* were isolated, and were named APCbmsn5Δ and FLPCbmsn5Δ respectively. Acid phosphatase activity was measured as described previously.³⁰⁾

Quantitative real-time PCR (qRT-PCR). Cells were grown aerobically to exponential phase in SM medium. Total RNAs was isolated from the cells with an RNeasy Mini Kit (Qiagen, Tokyo), followed by

on-column DNase digestion. cDNAs were synthesized from 1 µg of total RNA with Random Primers (Promega, Madison, WI) and ReverTra Ace (Toyobo, Osaka, Japan). After reverse transcription for 50 min at 42°C, samples were heated for 5 min at 99°C to terminate the reaction, and 0.5 µL of RNase H was added. qRT-PCR was performed in 20-µL mixtures in glass capillary tubes in a LightCycler (Roche Diagnostics, Tokyo). The PCR cycling reaction was performed with 1 × SYBR Premix Ex Taq (Takara Bio, Kyoto) according to the following parameters: first cycle, 10 min of denaturation at 95°C; second cycle with 40 repetitions, 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s (all temperature transitions, 20°C s⁻¹). The primers used in the reactions are listed in Table 1. As negative control, PCR was done without ReverTra Ace. The relative abundance of mRNAs was standardized against the levels for *PpACT1*.

Construction of a strain expressing *CbMsn5p* and YFP-fused protein. To visualize the localization of CbMsn5p, a strain expressing the CbMsn5p-YFP fusion protein was constructed as follows: First, the *CbMSN5* coding region and promoter region were amplified by PCR with primers SacI-MSN5-Fw and SbfI-MSN5-Rv, with genomic DNA as template. Then PCR was performed with primers PstI-YFP-Fw and NheI-YFP-Rv, with pTYT as the template.³⁰ The 5.4-kb SacI-SpeI fragment of pAPU, the 4.7-kb SacI-SbfI fragment of the promoter and coding regions of *CbMSN5*, and the 0.7-kb PstI-NheI fragment of the amplified YFP coding region were ligated to yield pCbMsn5-YFP. pCbMsn5-YFP was linearized with EcoRV and used to transform the *Cbmsn5Δura3* strain. The resulting strain was named CbMsn5p-YFP. Fluorescence microscopy and nuclear staining were performed as follows: CbMsn5p-YFP cells were harvested after exposure to stress conditions or non-stress conditions for 30 min, washed once, and fixed with 1 mL of 70% ethanol for 30 min at room temperature. The fixed cells were washed twice, resuspended in 150 µL of sterilized water, and stained with 150 µL of 0.125 µg/mL DAPI (4',6'-diamidino-2-phenylindole) solution. After 10 min of incubation, fluorescence was observed under a fluorescence-inverted microscope (IX70; Olympus, Tokyo), as described previously.¹⁶

Results

Cloning and characterization of the *C. boidinii* and *P. pastoris* homologs of *S. cerevisiae* *Msn5p*

A search of the *C. boidinii* and *P. pastoris* genome databases revealed the presence of putative homologs of *S. cerevisiae* Msn5p, designated CbMsn5p and PpMsn5p respectively. CbMsn5p and PpMsn5p consisted of 1,233 and 1,239 amino acid residues respectively, and were 58.3% identical and 84.3% similar to each other. The deduced amino acid sequence of CbMsn5p was 41.0% identical and 74.0% similar to that of ScMsn5p, and the deduced amino acid sequence of PpMsn5p was 39.7% identical and 75.1% similar to that of ScMsn5p.

Physiological characterization of the *C. boidinii* *msn5Δ* and *P. pastoris* *msn5Δ* strains

To analyze the physiological significance of CbMsn5p and PpMsn5p, we constructed *MSN5* gene-disrupted strains of *C. boidinii* and *P. pastoris* in which part of the ORF of the corresponding gene was replaced with the *Zeo^r* gene (Fig. 1; for details see "Materials and Methods"). We compared the growth of the wild-type and *MSN5* gene-disrupted strains on various carbon and nitrogen sources. The growth of the *C. boidinii* wild-type and *Cbmsn5Δ* strains on glucose or glycerol as sole carbon source was closely similar (data not shown). The *Cbmsn5Δ* strain exhibited retarded growth on methanol and on ethanol as sole carbon source as compared to the wild-type strain (Fig. 2A and B). In order to determine whether growth retardation was due to HCHO toxicity, the concentra-

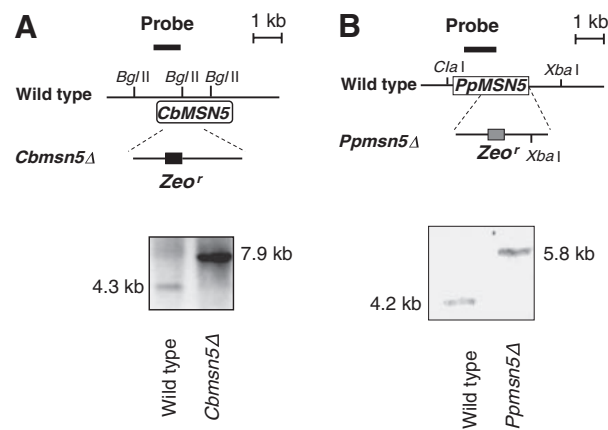


Fig. 1. One-Step Disruption of *MSN5* Genes in *C. boidinii* and *P. pastoris*.

A, Physical map and gene disruption of *CbMSN5*. B, Physical map and gene disruption of *PpMSN5*. The probes used for Southern analysis are marked by bold lines.

tions of HCHO in the culture medium of the wild-type and *Cbmsn5Δ* cultures grown on methanol were determined (Fig. 2A). With the wild-type strain, 0.33 mM HCHO accumulated after 26 h, but thereafter rapidly disappeared. With the *Cbmsn5Δ* strain, the HCHO level gradually increased until 30 h and then gradually decreased. These results suggest that CbMsn5p is involved in HCHO consumption during growth on methanol. When methylamine or choline, which are metabolized with formation of HCHO, was used as sole nitrogen source together with glucose as a carbon source, the *Cbmsn5Δ* strain also exhibited retarded growth as compared to the wild-type strain (Fig. 2C and D). In the case of *P. pastoris*, the *Ppmsn5Δ* strain grew as well on 1% methanol as the wild-type strain (Fig. 3A), but the *Ppmsn5Δ* strain showed retarded growth as compared to the wild-type strain on 3% methanol (Fig. 3B). This suggests that Msn5p plays an important role in methylotrophic yeasts when the cells are grown on substrates that result in the formation of HCHO, and that Msn5p is more critical for growth on methanol in *C. boidinii* than *P. pastoris*.

Msn5p was not involved in the transcriptional activation of methanol-inducible genes

To test whether disruption of *MSN5* affects the transcriptional activation of methanol-inducible genes, the expression levels of methanol-inducible genes were analyzed using a promoter-reporter assay for *C. boidinii* *AOD1* and *FLD1* and qRT-PCR analysis of *P. pastoris* *AOX1* and *FLD1*.

We constructed strains expressing the *ScPHO5* gene under the promoters for *CbAOD1* and *CbFLD1*. Both genes are induced not only by methanol but also by HCHO.¹²⁾ In glucose medium, expression of both genes was completely repressed, and both promoters exhibited basal levels of transcriptional activities in the wild-type and *Cbmsn5Δ* strains (Table 2). Promoter activities in the *Cbmsn5Δ* cells induced by both 0.7% methanol and 3 mM HCHO were higher than those of the wild-type strain (Table 2). However, when the cells were induced by 9 mM HCHO (the wild-type strain grew in a medium containing up to 9 mM HCHO), no reporter activity in

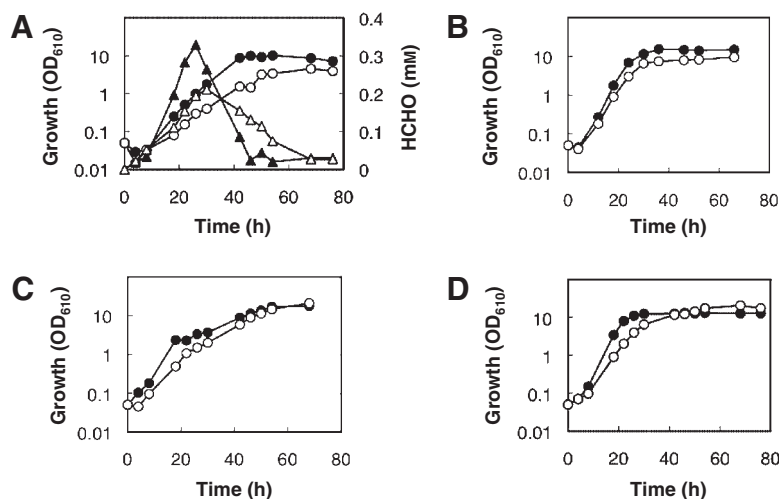


Fig. 2. Growth of the Wild-Type and *Cbmsn5Δ* Strains on Various Carbon and Nitrogen Sources, and the HCHO Concentration in the Medium. Cells were grown in YNB medium with the carbon and nitrogen sources indicated: A, 1% methanol; B, 1% ethanol; C, 2% glucose and 0.5% choline; and D, 2% glucose and 0.5% methylamine. Solid and hollow circles represent the growth of the wild-type and *Cbmsn5Δ* strains respectively; solid and hollow triangles represent the HCHO concentration in the medium with the wild-type and *Cbmsn5Δ* strains respectively.

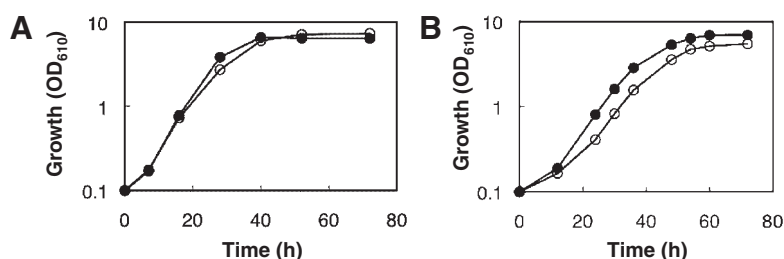


Fig. 3. Growth of the Wild-Type and *Ppmsn5Δ* Strains in YNB Medium with 1% Methanol (A) and 3% Methanol (B). Solid and hollow circles represent the wild-type and *Ppmsn5Δ* strains respectively.

Table 2. Levels of Acid Phosphatase Activity under the *AOD1* and *FLD1* Promoters in the *Cbmsn5Δ* Strain

Carbon source	Strain	Reporter activity ^a	
		<i>P_{AOD1}</i>	<i>P_{FLD1}</i>
Glucose	Wild type	0.79 ± 0.05	1.68 ± 0.09
	<i>Cbmsn5Δ</i>	1.09 ± 0.09	2.28 ± 0.04
Methanol	Wild type	24.5 ± 0.23	27.8 ± 1.32
	<i>Cbmsn5Δ</i>	66.0 ± 5.97	195 ± 11.0
HCHO (3 mM)	Wild type	57.9 ± 1.33	70.9 ± 2.81
	<i>Cbmsn5Δ</i>	117 ± 18.2	176 ± 2.81
HCHO (9 mM)	Wild type	1.74 ± 0.10	0.60 ± 0.25
	<i>Cbmsn5Δ</i>	N.D.	N.D.

^aThe level of acid phosphatase activity is expressed as U/OD₆₆₀ on the respective carbon source. The means of three independent experiments are shown. The wild-type and *Cbmsn5Δ* strains were incubated in the presence of 2% glucose, 0.7% methanol, 3 mM HCHO, or 9 mM HCHO for 8 h. N.D., Activity could not be detected.

the *Cbmsn5Δ* strain was detected, because the cells were dead at 9 mM HCHO. Similar results were observed for the expression levels of the *PpAOX1* and *PpFLD1* genes in the cells induced by methanol, as determined by qRT-PCR analysis (Table 3). This indicates that Msn5p is not involved in the transcriptional activation of methanol/HCHO-inducible genes, and participates in a defense mechanism against HCHO. In the Msn5p-depleted strains, the intracellular level of HCHO might be higher than in the wild-type strain, and in that case, the accumulated HCHO more strongly induces the expression of HCHO-inducible genes.

Table 3. Transcriptional Levels of *PpAOX1* and *PpFLD1* in the *Ppmsn5Δ* Strain

Carbon source	Strain	Relative transcriptional level	
		<i>PpAOX1</i>	<i>PpFLD1</i>
Methanol	Wild type	100%	100%
	<i>Ppmsn5Δ</i>	143%	201%

The *P. pastoris* wild-type and *Ppmsn5Δ* strains were incubated in the presence of 0.7% methanol for 3 h. The relative abundance of mRNAs was standardized against the level of *PpACT1*.

Msn5p was involved in the defense mechanism against HCHO, but not against H₂O₂

To elucidate the functional consequences of depletion of Msn5p on the HCHO or H₂O₂ stress response system, the *Cbmsn5Δ* and *Ppmsn5Δ* strains were plated on YNB-glucose agar plates containing an appropriate concentration of HCHO or H₂O₂. The *Cbmsn5Δ* strain exhibited higher sensitivity to HCHO than the wild-type strain (Fig. 4A). On the other hand, the *Cbmsn5Δ* strain was observed to become hyper-resistant to 1 mM H₂O₂. Similarly, the *Ppmsn5Δ* strain was more sensitive to HCHO, but less sensitive to H₂O₂ (Fig. 4B) as compared with the wild-type strain. This suggests that Msn5p is involved in the defense mechanism against HCHO, but not against H₂O₂.

Subcellular localization of CbMsn5p-YFP

In *S. cerevisiae*, ScMsn5p is concentrated in the nuclei under non-stress conditions, but diffuses into the

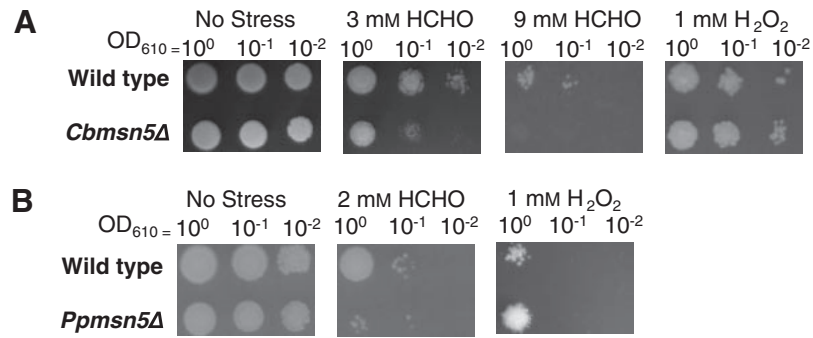


Fig. 4. Cell Viability in Medium Containing HCHO or H₂O₂.

A, The *C. boidinii* wild-type and *Cbmsn5Δ* strains were incubated in YNB-glucose medium containing 3 mM HCHO, 9 mM HCHO, or 1 mM H₂O₂, and then 10-fold serial dilutions were spotted on YNB-glucose agar plates, which were incubated for 2 d at 28 °C. B, The *P. pastoris* wild-type and *Ppmsn5Δ* strains were incubated in YNB-glucose medium containing 2 mM HCHO and 1 mM H₂O₂, and then 10-fold serial dilutions were spotted on YNB-glucose agar plates, which were incubated for 2 d at 28 °C.

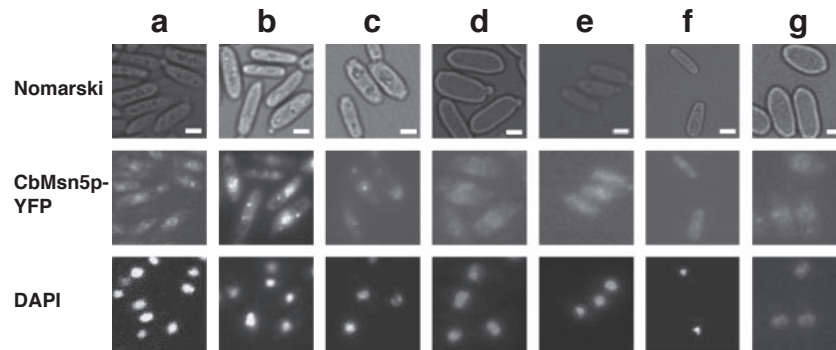


Fig. 5. Subcellular Localization of CbMsn5p-YFP.

CbMsn5p-YFP was expressed under the control of the native promoter in the *Cbmsn5Δ* strain. CbMsn5p-YFP expressing cells were incubated on YNB medium containing various carbon sources, and YFP fluorescence and DAPI-stained nuclei were observed using fluorescence microscopy. a, 2% glucose (at mid-log phase); b, 1% methanol (mid-log phase); c, 1% methanol (30 min incubation); d, 2% glucose with 3 mM HCHO (30 min incubation); e, 2% glucose with 9 mM HCHO (30 min incubation); f, 10% ethanol (30 min incubation); g, 2% glucose with 1 mM H₂O₂ (30 min incubation). Bar, 1 μm.

cytosol upon exposure to various stresses, *e.g.*, ethanol, heat, starvation, and severe oxidative stress.²¹⁾ To investigate the subcellular localization of CbMsn5p, we expressed a recombinant CbMsn5p tagged with YFP at the C-terminus (Msn5p-YFP) under the control of the native promoter in the *Cbmsn5Δ* strain.

The growth rate of the *Cbmsn5Δ* strain expressing Msn5p-YFP on glucose with 3 mM HCHO was similar to that of the wild-type strain (data not shown), indicating that fusion of the YFP protein to Msn5p did not interfere with its native function. When cells expressing CbMsn5p-YFP were grown on glucose or methanol to mid-log phase, or when the cells were exposed to 1% methanol for 30 min, the fluorescence of CbMsn5p-YFP was partially concentrated in the nucleus, as shown by staining with DAPI (Fig. 5a, b, and c). When the cells were exposed to 3 mM HCHO, CbMsn5p-YFP accumulated in the nucleus (Fig. 5d). However, when the cells were exposed to 6–9 mM HCHO, CbMsn5p-YFP diffused into the cytosol (Fig. 5e). The distribution of ScMsn5p has been reported to be correlated with the concentration of ethanol.²¹⁾ CbMsn5p-YFP diffused into the cytosol when the cells were exposed to 10% ethanol (Fig. 5f), but CbMsn5p-YFP accumulated in the nucleus when the cells were exposed to 1 mM H₂O₂ (Fig. 5g). When the cells were exposed to 5 mM H₂O₂, CbMsn5p-YFP still accumulated in the nucleus (data not shown). This suggests that CbMsn5p responds to HCHO stress and ethanol stress but not to H₂O₂ stress.

Discussion

In yeast methanol metabolism, two toxic compounds, HCHO and H₂O₂, are generated by alcohol oxidase. In previous studies, we determined the physiological functions of each of the enzymes involved in the detoxification of these compounds and the importance of the GSH redox system in methylotrophic yeasts.³⁾ The GSH redox system is regulated by oxidative stress regulator Yap1p, and a *Ppyap1Δ* strain showed a prolonged lag phase during growth on methanol, but this strain did not show sensitivity to HCHO.^{15,16)} Thus it was clear that the cellular response to ROS derived from H₂O₂ is regulated by transcription factor Yap1p, but the molecular mechanism of the cellular response to HCHO remained unknown.

In this study, we focused on Msn5p, a nuclear exporter of the importin-β family, in methylotrophic yeasts. *MSN5* was originally isolated as a multicopy suppressor in *S. cerevisiae* mutants defective in Snf1p, which is a protein kinase and a key regulator of cell growth and a metabolic sensor.^{31,32)} Although multiple functions for Msn5p have been elucidated, there has been no report as to whether Msn5p participates in the HCHO resistance mechanism. In the present study, we found that Msn5p in the methylotrophic yeasts was involved in the cellular defense mechanism against HCHO by gene disruption analysis and observation of the subcellular localization of Msn5p.

In *S. cerevisiae*, the subcellular localization of Msn5p is controlled by stress and carbon source in a Snf1p-dependent manner.^{21,33} ScMsn5p is concentrated in the nuclei under non-stress conditions, but appears in the cytoplasm under stress conditions, such as exposure to ethanol, heat, starvation, or oxidative stress.²¹ YFP-tagged CbMsn5p was diffuse in the cytoplasm when the cells were treated with high concentrations of HCHO (6–9 mM) and ethanol (10%), but fluorescence was concentrated in the nuclei following treatment with H₂O₂ (1–5 mM). Taken together with the results as to the sensitivity of the *msn5Δ* strains to HCHO and H₂O₂, it is clear that Msn5p participates in the response to HCHO stress but not directly in H₂O₂ stress in the methylotrophic yeasts. Interestingly, the behavior of CbMsn5p-YFP was similar when the cells were exposed to HCHO and ethanol, indicating that the cellular responses to HCHO and ethanol stress might be regulated in part by the same pathway. In *S. cerevisiae*, molecular responses to ethanol and acetaldehyde stress have been studied,^{34,35} but no effect of acetaldehyde or HCHO on ScMsn5p has been reported.

Msn5p mediates transcriptional regulation by exporting transcription factors, such as Mig1p and Pho4p, in *S. cerevisiae*.^{36,37} Hence it is possible that Msn5p in the methylotrophic yeasts functions in the transcriptional regulation of genes encoding methanol metabolism. However, our results indicate that Msn5p is not involved in the transcriptional activation of *AOD1* and *FLD1* in methylotrophic yeasts (Tables 2 and 3). The transcription levels of these genes in the *msn5Δ* strains were significantly higher than those in the wild-type strain. Their promoters might be more strongly activated by higher levels of HCHO by an unknown mechanism in the *msn5Δ* strain.

In addition to nuclear export, ScMsn5p mediates the nuclear import of replication protein A (RPA), which is required for multiple aspects of DNA metabolism, including DNA replication, DNA repair, and recombination.^{22,38–40} It is well known that HCHO is non-specifically reactive with nucleic acids, and that exposure to HCHO results in damaged DNA or inhibition of DNA repair. Hence we speculate that HCHO temporarily accumulates in cells, causing toxic effects on the DNA and/or DNA repair, and that RPA is required to protect cells from HCHO toxicity. Disruption of *MSN5* can result in a reduced rate of localization of RPA in the nucleus and increased cell sensitivity to HCHO. To substantiate this hypothesis, further experiments are needed.

Our findings perhaps shed light on the details of the defense mechanism against HCHO, and might help to employ the methylotrophic yeast efficiently to produce foreign protein at high HCHO concentrations. To our knowledge, this is the first report of this new role for Msn5p, which is indirectly or directly involved in the response to HCHO stress in methylotrophic yeasts.

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