Journal of Bioscience and Bioengineering
VOL. 128 No. 6, 697–703, 2019

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Xylene causes oxidative stress and pronounced translation repression in *Saccharomyces cerevisiae*

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Received 19 March 2019; accepted 30 May 2019
Available online 22 June 2019

Organic solvent-resistant microorganisms are strongly desired for efficient fermentative production of hydrophobic substances in water-organic solvent two-phase systems. To improve organic solvent-resistance of microorganisms, a better understanding of the effects of organic solvents on microbial cells and cellular responses to organic solvents is essential. So far, various bacteria have been studied for their response mechanisms against organic solvents and improvement of their resistance to organic solvents. On the other hand, limited information is available on the effects of organic solvents on eukaryotic microorganisms. We herein examined the physiological effects of xylene, one of representative organic solvents, on the budding yeast *Saccharomyces cerevisiae*. We found that xylene induced fragmentation of mitochondria and the nuclear accumulation of Yap1, an oxidative stress responsive transcription factor, followed by the transcriptional activation of its target genes, *GPX2* and *TRX2*, in yeast cells treated with xylene. These findings indicate that xylene caused oxidative stress in yeast cells. However, treatment with 0.03% (v/v) or more of xylene severely repressed the translation activity of yeast cells. Therefore, the expected protein synthesis of Yap1-target genes was not observed despite the transcriptional activation in cells treated with 0.03% (v/v) xylene. This is the first report on the inhibitory effects of xylene on bulk translation activity and provides novel insights into the toxicity of xylene.

[**Keywords:** Xylene; Translation repression; Processing bodies; Stress granules; Oxidative stress; *Saccharomyces cerevisiae*]

Fermentative production of useful substances using microorganisms is more energy intensive and exerts less environmental burden than other production methods, such as conventional chemical reactions. In the production of substances using microorganisms, when the substrates or the products are hydrophobic, fermentation may be carried out in water-organic solvent two-phase systems with an aqueous solution and an organic solvent (1,2). However, growth and fermentation of microorganisms are often inhibited in the presence of organic solvents. Therefore, organic solvent-resistant microorganisms are in great need for efficient fermentative production. Based on these backgrounds, many bacteria including *Escherichia coli* and *Pseudomonas* species have been studied for their response mechanisms against organic solvents and the improvement of their resistance against such solvents (2–8). On the contrary, information on the response mechanisms of eukaryotic microorganisms to organic solvents is still very limited, although several efforts to improve organic solvent tolerance of yeast cells have been reported (9–13).

Xylene, one of the standard organic solvents, is an aromatic hydrocarbon produced in large quantities that is widely used in industrial plants. It is also one of the causative substances for sick building syndrome. Chronic exposure to xylene causes various health issues, such as irritation in the respiratory systems and eyes, skin erythema, and scaling of skin (14–17). Although the influence of xylene on the human body and the specific symptoms it causes are well-known, its action mechanisms at the cellular or molecular level have not been fully clarified. Moreover, very little information is available about the physiological effects of xylene on eukaryotic microorganisms.

In this study, we analyzed the susceptibility and cellular responses to xylene of the budding yeast *Saccharomyces cerevisiae*, a very useful eukaryotic microorganism in fermentative production. Exposure to xylene caused oxidative stress and activation of Yap1, an oxidative stress-responsive transcription factor (18), followed by the transcriptional activation of its target genes in yeast cells. However, the translation activity was severely repressed in the presence of 0.03% (v/v) or more of xylene, and the ultimate expression (protein synthesis) of the Yap1-target genes, whose mRNA levels were elevated, was not reached. We demonstrated the inhibitory effects of xylene on bulk translation activity for the first time, and our data might provide a clue regarding the effects of xylene on human translation activity.

MATERIALS AND METHODS

Strains and medium *S. cerevisiae* strains BY4741 (*MAT***a** *his3*Δ*1 ura3*Δ*0 leu2*Δ*0 met15*Δ*0*) and DY (*MAT*α *his3 can1-100 ade2 leu2 trp1 ura3::*(3xSV40AP1-LacZ)) (18) were used in the present study. Cells were cultured in 50 ml of synthetic defined (SD) medium (2% glucose and 0.67% yeast nitrogen base without amino acids) with appropriate supplements of amino acids and bases, at 28 °C with reciprocal shaking (120 rpm) in Erlenmeyer flasks (300 ml). Cells were harvested during exponential growth at an optical density at 600 nm (OD_{600}) of 0.5 and were treated with stress of xylene or hydrogen peroxide (H_2O_2).

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2019.05.016

TABLE 1. List of primer pairs for quantitative PCR.

Gene	Primer sequences
GPX2	Forward: 5′-CTTCACGCCGCAGTA TAAAGA-3′
	Reverse: 5′-CCTGCTTCCCGAACTGA TTAC-3′
TRX2	Forward: 5′-TGGATGTTGATAAGTC TCAGATG-3′
	Reverse: 5′-CCTCCTTACCGCCCTTG TAG-3′
ACT1	Forward: 5′-TTGGATTCCGGTGATGG TGTTACT-3′
	Reverse: 5′-TGAAGAAGA TTGAGCAGCGGTTTG-3′

Stress treatment For the stress treatment, yeast cells were collected by centrifugation and were re-suspended in fresh SD medium to obtain an initial OD_{600} value of 0.1. Cells were treated with various concentrations of xylene or H_2O_2 at 28 °C with shaking at 120 rpm. Technical grade xylene (>90% mixture of the three isomers of xylene) was purchased from Nacalai Tesque (Kyoto, Japan; 36612-35), and stock solution of 10% (v/v) xylene was prepared in dimethyl sulfoxide (DMSO). To assess the susceptibility to xylene, cells treated with xylene were diluted with 100 mM potassium phosphate buffer (pH 6.8) and plated on yeast peptone dextrose (YPD) agar plates (2% glucose, 2% peptone, 1% yeast extract, and 2% agar, pH 5.5), which were incubated at 28 °C for two days to calculate colony-forming units (CFUs). The survival of cells without xylene treatment was taken as 100%.

Plasmids The integration-type plasmid YIp-*GPX2-FLAG* was constructed to estimate the protein levels of Gpx2. A part of the open reading frame (ORF) of *GPX2* was amplified by a polymerase chain reaction (PCR) using the primers 5′-GCAAGGTCTAGAAAGGCGAATCTTTTAAGTTTGAC-3′ and 5′-GACAAACTCGAGATTTACTTAACAGGCTTTGG-3′. The amplicon was cloned into the *Xba*I/*Xho*I sites of YIp-*SSA4*-GFP (19) after digestion with *Xba*I/*Xho*I to construct YIp-*GPX2*-GFP. A 0.5-kbp fragment encoding a FLAG tag sequence, stop codon, and the 3′-flanking region of *GPX2* was amplified using the primers 5′-GCAAGCTCGAGCTGACTACAAGGATGACGATGACAAGTGATATCTTTGTCTATAAT-3′ and 5′-GGCTTGGTACCAGATGACGTATACGAATTAGTGCGTGATG-3′. The amplicon was digested with *Xho*I/*Kpn*I and cloned into the *Xho*I/*Kpn*I sites of YIp-*GPX2-GFP* to construct YIp-*GPX2-FLAG*. To integrate the *GPX2-FLAG* at the chromosomal *GPX2* locus, YIp-*GPX2-FLAG* was linearized through its digestion with *Eco*RI and was then introduced into yeast cells. Plasmids used for observing mitochondrial morphology (mito-GFP: pVT100U-mtGFP) (20) and Yap1-localization (GFP-Yap1: pRS cup1 cp-GFP-YAP1) (21) were provided by Dr. K. Okamoto and Dr. S. Kuge, respectively. The integration-type plasmids of Dhh1-GFP, Dcp2-GFP, Pab1-GFP, and Pbp1-GFP were described previously (22).

Gene expression analysis The relative mRNA levels of the Yap1-target genes, *TRX2* and *GPX2*, were assessed using quantitative PCR (qPCR). The qPCR method has been described previously (23). The sequences of the DNA primers for qPCR are listed in Table 1. Polysome profile analysis and Western blotting analysis were performed as previously described (22–24). The polysome profile analysis was carried out by 10–50% sucrose gradient separation of ribosome fractions using the methods of Inada and Aiba (25). A monoclonal anti-FLAG M2 antibody (F1804; Sigma–Aldrich, St. Louis, MO, USA) was used to monitor the levels of Gpx2-FLAG. A monoclonal anti-PGK antibody (A-6457; Molecular Probes, Eugene, OR, USA) was used to monitor the levels of Pgk1, which was used as a loading control. An anti-mouse IgG, HRP-linked antibody (7076S, Cell Signaling Technology, Danvers, MA, USA) was used as the secondary antibody. The Yap1-dependent reporter assay using the 3xSV40AP1-*lacZ* gene was described previously (26).

Microscopic analysis and intracellular oxidation levels A Leica AF6500 fluorescence microscope system (Leica Microsystems Vertrieb GmbH, Wetzlar, Germany) was used for the microscopic analysis. Cells treated with xylene were immediately observed without fixation. Levels of intracellular oxidation were measured using an oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) (D399; Molecular Probes) as described previously (19,27). The percentages of cells containing fragmented mitochondria, GFP-Yap1 accumulated in the nucleus, processing bodies (PBs), or stress granules (SGs) were calculated by monitoring 100 cells under each condition, and experiments were repeated three times (a total of 300 cells were examined). Data are represented as means ± standard deviation (SD).

RESULTS

Yeast susceptibility to xylene First, we examined the toxicity of xylene to yeast cells. As shown in Fig. 1, the number of living cells was decreased upon treatment with xylene in a concentration-dependent manner, and more than half of the cells were killed upon treatment with 0.04% (v/v) xylene within 45 min. These results clearly indicate that xylene is toxic for yeast cells and more than 0.04% (v/v) xylene quickly prevents their survival, suggesting that treatment with less than 0.04% (v/v) xylene would be suitable to monitor and examine cellular responses to xylene.

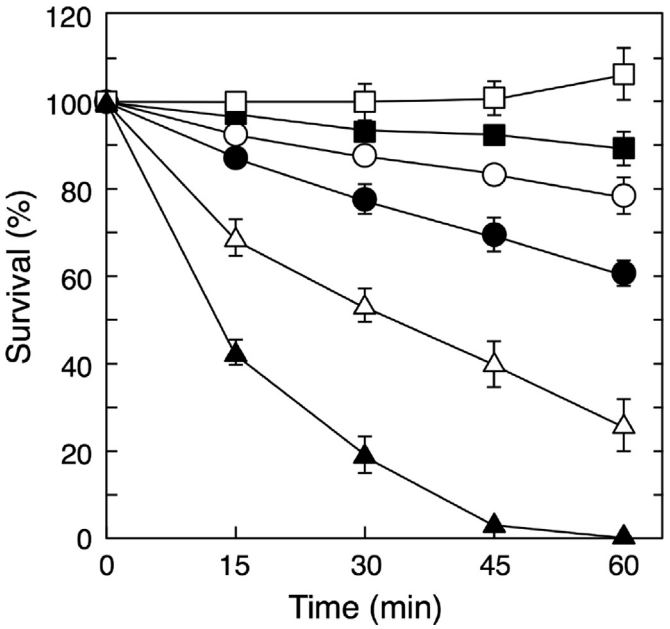

FIG. 1. Yeast susceptibility to xylene. Yeast cells in an exponential phase of growth were treated with xylene for the indicated period in SD medium. The relative survival rate was calculated as colony-forming units (CFU). The survival of cells without the stress treatment was taken as 100%. Data are given as the means ± SD (n = 3). The concentrations of xylene examined are as follows: 0% (v/v) (open squares), 0.01% (v/v) (closed squares), 0.02% (v/v) (open circles), 0.03% (v/v) (closed circles), 0.04% (v/v) (open triangles), and 0.05% (v/v) (closed triangles).

Xylene exposure induced oxidative stress in yeast cells Since various volatile organic compounds cause oxidative stress (17,28–30), we first examined whether xylene also causes oxidative stress in yeast cells. The oxidative stress often damages mitochondria and causes drastic changes in mitochondrial morphology. Mitochondria usually maintain a tubular shape because of the balance between the two opposing processes, fission and fusion. However, their fragmentation is induced under oxidative stress conditions due to increased fission (31–34). Therefore, using mito-GFP, we examined whether xylene causes the fragmentation of mitochondria (20). We found that the percentage of cells with fragmented mitochondria was significantly increased by the treatment with more than 0.02% (v/v) xylene (Fig. 2A). Treatment with 0.03% (v/v) xylene induced the comparable levels of fragmented mitochondria with 0.4 mM H_2O_2. These results suggest that xylene induced oxidative stress in yeast cells.

To confirm the induction of oxidative stress, we next examined intracellular levels of reactive oxygen species (ROS) using an oxidant-sensitive probe, H_2DCFDA (19,27,35). We observed a remarkable elevation in ROS levels after the treatment with xylene. Moreover, similar results were obtained after the treatment with H_2O_2 as well (Fig. 2B). These results imply that intracellular accumulation of ROS was caused by the treatment with xylene.

Expression of Yap1-target genes under xylene stress Although the assay using H_2DCFDA suggested that xylene may cause ROS accumulation (Fig. 2B), we could not exclude

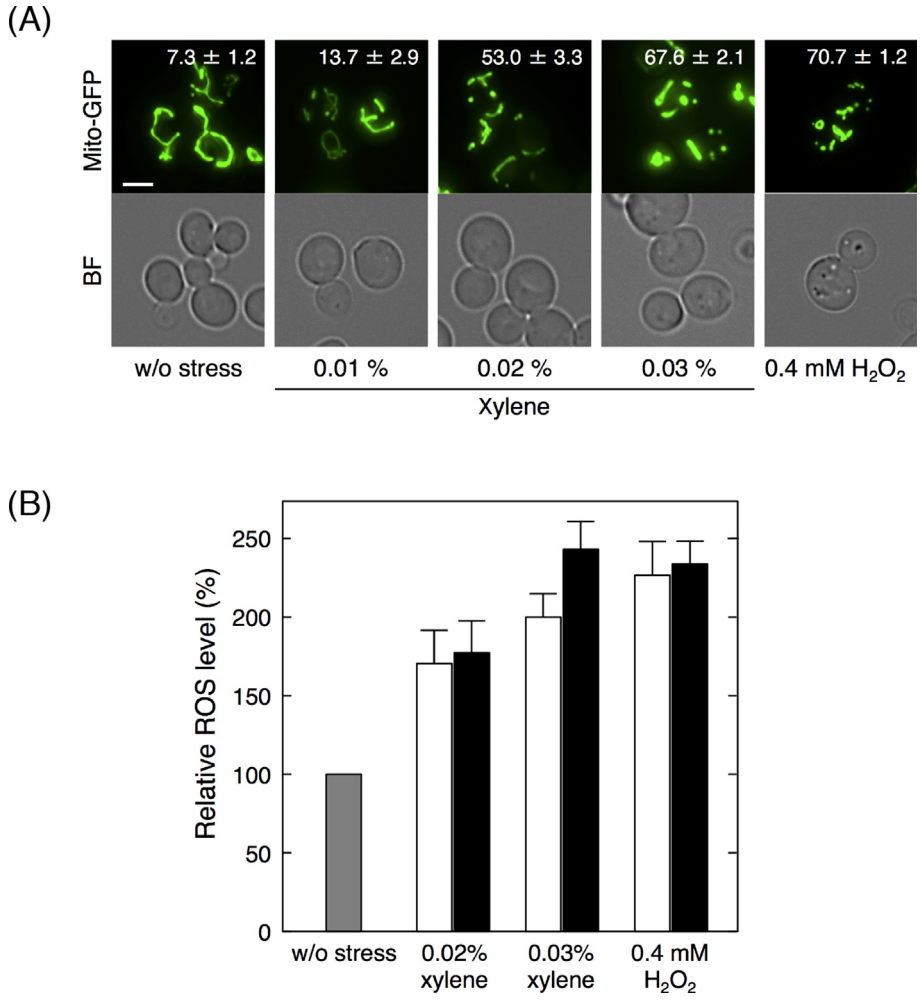

FIG. 2. The oxidative stress response was induced by xylene exposure. (A) Cells expressing the mitochondria-targeted green fluorescent protein (mito-GFP) were treated with xylene or H_2O_2 for 15 min. Representative microscopic images are shown in the figure. The white bar indicates 5 μm. Numbers in the panels indicate the mean ± SD percentage of cells containing the fragmented mitochondria. (B) Intracellular oxidation levels in yeast cells were examined using H_2DCFDA. Cells were treated with xylene or H_2O_2 for 15 min (open bars) or 30 min (closed bars). The fluorescence intensity of cells without the stress treatment was taken as 100%. Data are given as the means ± SD ($n = 3$).

a possibility that xylene directly interacts with and oxidizes the oxidant-sensitive probe. As another way to confirm that xylene causes oxidative stress in yeast cells, we next focused on Yap1, an oxidative stress responsive transcription factor of yeast (18). Yap1 usually distributes in the cytoplasm via nuclear export under non-stressed conditions but accumulates in the nucleus under oxidative stress and activates the transcription of Yap1-target genes (18,36–38). Xylene as well as H_2O_2 caused the nuclear accumulation of GFP-Yap1, and more than 70% of the cells accumulated GFP-Yap1 in the nucleus after 60-min treatment with 0.02 or 0.03% (v/v) xylene (Fig. 3A). These results strongly suggest that treatment with xylene induced oxidative stress and the activation of Yap1.

To verify the activation of Yap1 by xylene, we next examined the expression of Yap1-target genes under xylene stress. *GPX2* (encoding a phospholipid hydroperoxide glutathione peroxidase) and *TRX2* (encoding a cytoplasmic thioredoxin) are representative Yap1-target genes and it was clarified that their transcriptional activation is caused by oxidative stress in the Yap1-dependent manner (18,37,39). We performed qPCR analysis and observed that mRNA levels of *GPX2* and *TRX2* were significantly increased by xylene and H_2O_2 treatment (Fig. 3B). These results confirmed that the activation of Yap1 was caused by xylene.

To examine whether protein synthesis of Yap1-target genes is enhanced by xylene, we further performed a Yap1-dependent reporter assay using the 3xSV40AP1-*lacZ* gene, the transcription of which is regulated solely by Yap1 through triple Yap1-binding sites (3xSV40AP1) (18,26,37). Treatment with 0.02% (v/v) xylene and 0.4 mM H_2O_2 led to the increased activities of β-galactosidase derived from 3xSV40AP1-*lacZ* (Fig. 3C). However, treatment with 0.03% (v/v) xylene did not cause an elevation of β-galactosidase activity. We additionally performed Western blot analysis to examine whether the protein synthesis of Gpx2 is enhanced by xylene and found that cells treated with 0.02% (v/v) xylene showed increased levels of the Gpx2 protein (Fig. 3D). However, cells treated with 0.03% (v/v) xylene did not increase the level of Gpx2 protein despite the increased levels of *GPX2* mRNA (Fig. 3B and D), indicating that increased mRNA expression (*GPX2*) did not result in the expected increase in protein expression (Gpx2) in cells treated with 0.03% (v/v) xylene.

Xylene caused pronounced translation repression Since the expected protein synthesis was not observed in cells treated with 0.03% (v/v) xylene, we speculated that bulk translation repression was induced by 0.03% (v/v) xylene. In eukaryotic cells, pronounced translation repression is often accompanied by the formation of cytoplasmic mRNP granules, such as PBs and SGs. They

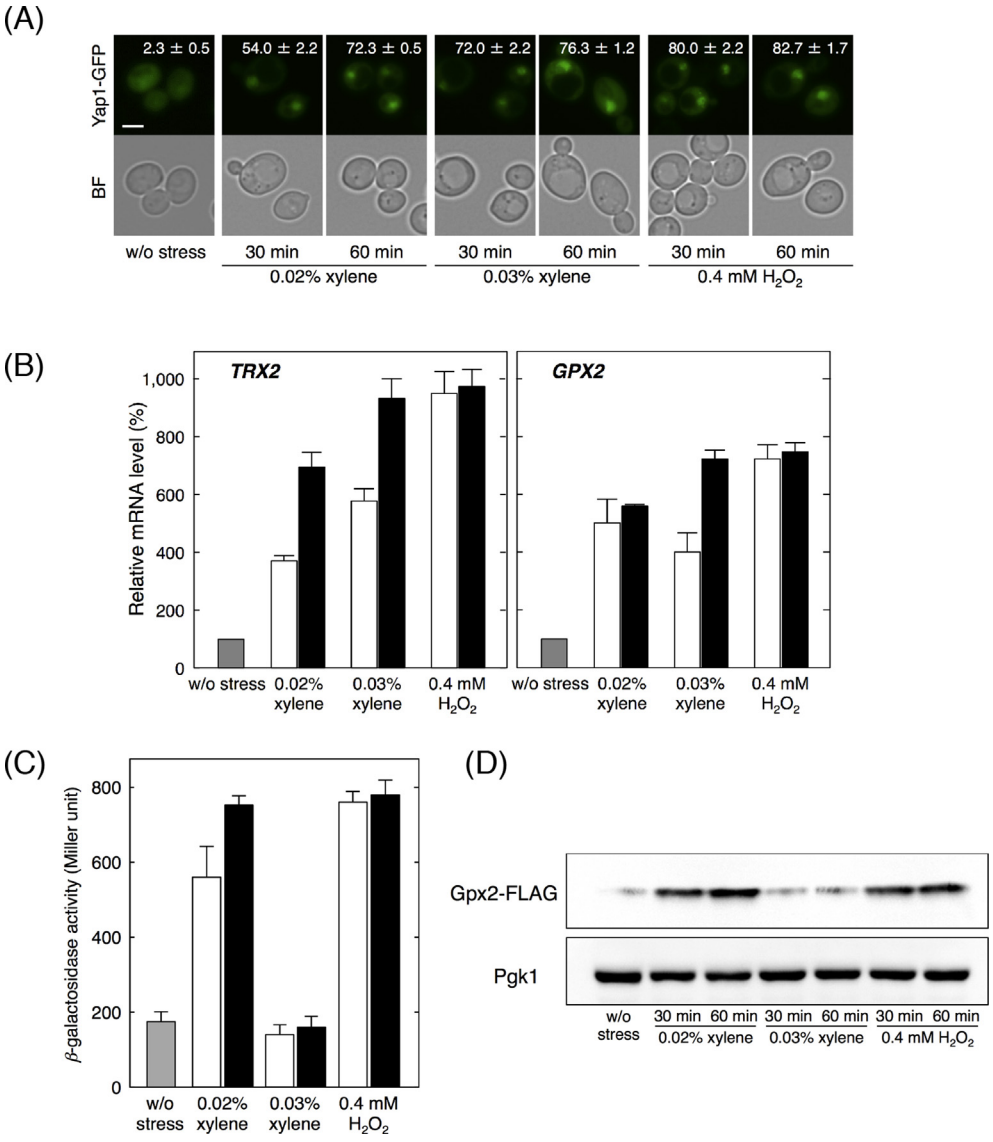

FIG. 3. Effects of xylene on the expression of Yap1-target genes. (A) Cells expressing GFP-Yap1 were treated with xylene or H_2O_2 for 30 or 60 min. Representative microscopic images are shown in the figure. The white bar indicates 5 μm. Numbers in the panels indicate the mean ± SD percentage of cells showing the nuclear accumulation of GFP-Yap1. (B) The mRNA levels of *TRX2* and *GPX2* in cells treated with xylene or H_2O_2 for 30 min (*white bars*) or 60 min (*black bars*) were assessed through qPCR. The mRNA levels of *TRX2* and *GPX2* were normalized to that of *ACT1*. Data are shown as the mean ± SD ($n = 3$). (C) Cells carrying the 3xSV40AP1-*lacZ* gene were treated with xylene or H_2O_2 for 30 min (open bars) or 60 min (closed bars), and β-galactosidase activity was then assayed. Results represent the mean ± SD. ($n = 3$). (D) Cells carrying a FLAG-tagged chromosomal copy of *GPX2* were treated with xylene or H_2O_2. Protein levels of Gpx2-FLAG were evaluated by a Western blot analysis. Pgk1 was used as a loading control.

are composed of non-translating mRNAs and various RNA-associated proteins and are considered to be involved in the regulation of translation repression under various stress conditions (40,41). In yeast cells, the formation of PBs and SGs is induced by glucose depletion and severe ethanol stress, which cause pronounced translation repression (42–46).

To confirm the induction of pronounced translation repression by xylene, we examined whether xylene induces the formation of PBs and SGs. In this study, we used Dhh1-GFP and Pab1-GFP as a representative PB marker and a SG marker, respectively (22,47). We found that 0.03% (v/v) xylene as well as glucose depletion clearly induced the formation of PBs and SGs (Fig. 4). We also observed the formation of cytoplasmic granules of another SG marker (Pbp1-GFP) and PB marker (Dcp2-GFP) under 0.03% (v/v) xylene stress (data are not shown). These results imply that 0.03% (v/v) xylene caused translation repression in yeast cells.

Moreover, to see the effects of xylene on the overall protein synthesis, we further analyzed the bulk translation activity by assessing the polysome profiles (24,46,47). Although cells treated with 0.02% (v/v) xylene showed a lower polysome ratio than cells without stress treatment, they still formed an adequate amount of polysome (approximately 40%), indicating the retention of sufficient translation activity (Fig. 5). On the contrary, cells treated with 0.03% or 0.04% (v/v) xylene showed a significant reduction in the polysome fraction and a concomitant increase in the 40S and 80S fractions. This indicates that pronounced repression of bulk translation activity was caused by more than 0.03% (v/v) xylene. The significant reduction in the polysome fraction continued at least for 6 h in the presence of 0.03% (v/v) xylene. These results clearly indicate that more than 0.03% (v/v) xylene caused the constitutive repression of bulk translation activity in yeast cells.

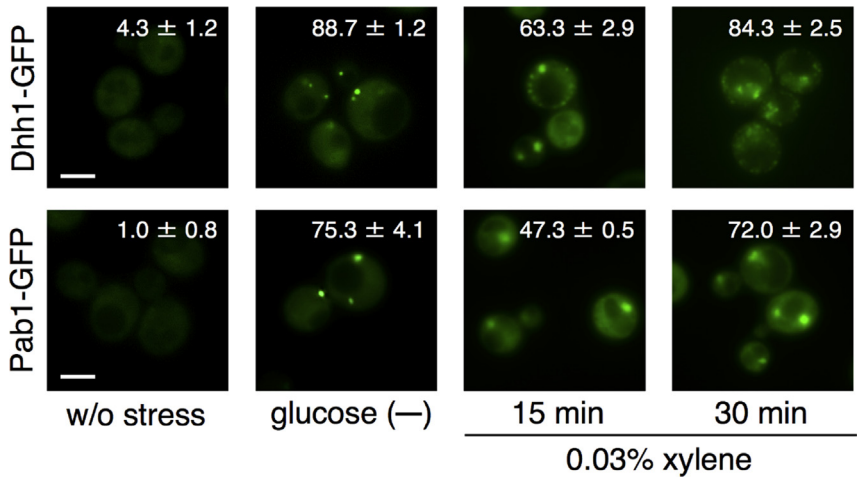

FIG. 4. Formation of the cytosolic mRNP granules in cells treated with xylene (15 or 30 min) or cells stressed through glucose depletion (15 min). The assembly of PBs and SGs was examined using Dhh1-GFP and Pab1-GFP, respectively. Representative pictures are shown in the figure. The white bar indicates 5 μm. Numbers in the panels indicate the mean ± SD percentage of cells containing PBs or SGs.

DISCUSSION

The results of the current study demonstrated that xylene induced ROS accumulation and activation of Yap1 in yeast cells. Many studies reported that various volatile organic compounds such as toluene and benzene induce oxidative stress in mammalian cells and insect cells (28–30,48,49). In the case of xylene, Salimi et al. (17) reported that xylene induced ROS generation and mitochondrial damage in human leukemia cells and lymphocytes. Therefore, it appears feasible that xylene causes ROS accumulation and oxidative stress even in yeast cells. Nishida-Aoki et al. (12) reported that isooctane, another representative organic solvent, induced the generation of ROS in yeast cells via mitochondrial damage. Similar to isooctane, xylene might damage mitochondria first and then lead to the emission of ROS and mitochondrial fragmentation in yeast cells. This speculation seems to be persuasive because xylene damaged mitochondria in human cells (17) and caused changes in mitochondrial morphology in yeast cells (Fig. 2A).

We also found that severe xylene stress [>0.03% (v/v)] rapidly caused the repression of bulk translation activity accompanied by the formation of PBs and SGs. Therefore, yeast cells could not synthesize Gpx2 protein despite the transcriptional activation of *GPX2* in the presence of 0.03% (v/v) xylene. It is presumable that other Yap1-target genes can also be activated at the level of transcription but not at the level of translation under severe xylene stress. Therefore, it is likely that yeast cells cannot reinforce antioxidant systems under severe xylene stress.

It is known that high salinity stress (1 M NaCl) causes pronounced but transient translation repression and yeast cells can recover the translation activity within 2 h, demonstrating that yeast cells can adapt to the high salinity stress (50). In contrast, the pronounced translation repression continued at least for 6 h in the presence of 0.03% (v/v) xylene (Fig. 5). These results might suggest the greater difficulty faced by yeast cells to regain translation activity under severe xylene stress, compared to stress induced by 1 M NaCl. Therefore, once sufficient xylene penetrates into yeast cells and disturbs the activity of translation, it might be almost impossible for yeast cells to recover from the damages caused by xylene because of the deficiency of translation activity. Since the cells cannot improve stress tolerance through the synthesis of new proteins, the ability of the cells to exclude xylene would be crucial for the survival of yeast cells under xylene stress. Nishida et al. (51) reported that overexpression of ATP-binding cassette (ABC) transporters, such as Pdr10 and Snq2, conferred resistance to organic solvents in yeast cells. In-advance reinforcement of the activity of ABC drug pumps, which eliminate xylene from the cells, seems to be a reasonable and practical strategy for the improvement of xylene tolerance in yeast cells.

As a subject for future analysis, the mechanisms through which xylene inhibits protein translation should be examined. Since oxidative stress has little or no effect on the induction of SG-formation and translation repression in *S. cerevisiae* cells (41,42,52), it is conceivable that some factors other than oxidative stress might cause translation repression in the presence of xylene. It is well known that various organic solvents destabilize protein

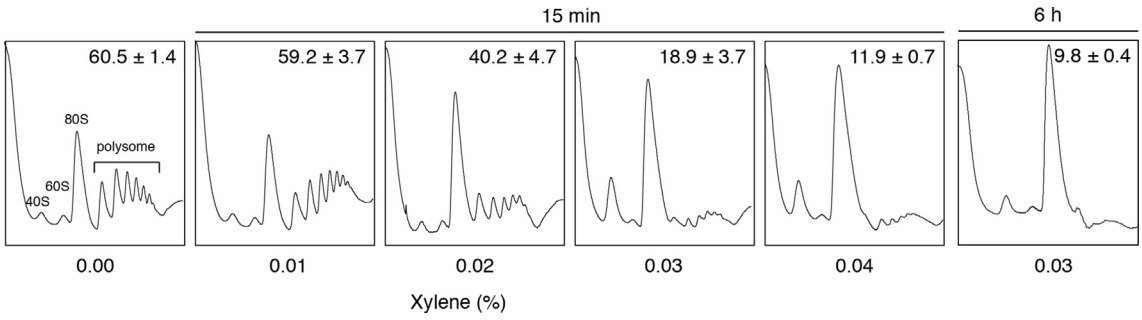

FIG. 5. Xylene caused the pronounced translation repression. The polysome profiles of cells treated with xylene for 15 min or 6 h are shown. The polysome, 40S (small ribosomal subunit), 60S (large ribosomal subunit), and 80S (monosome) peaks are labeled. The polysome ratio was calculated and is shown as the mean ± SD ($n = 3$).

conformation (53–55). Although 0.03% (v/v) xylene seems to be too low for inducing protein denaturation (54,55), xylene might destabilize the conformation of ribosome components in yeast cells. Since yeast ribosome and mammalian ribosome have many things in common in their basic structures and characteristics (56,57), it is tempting to speculate that the translation activity in human cells might also be disturbed by xylene. Effects of xylene on translation activity in human cells should be examined as another interesting subject for future analysis.

ACKNOWLEDGMENTS

We thank Shusuke Kuge (Tohoku Medical and Pharmaceutical University) and Koji Okamoto (Osaka University) for providing strains and plasmids. This study was supported by the Japan Society for the Promotion of Science (grant number 17H03795 to S.I.). The authors declare that they have no conflict of interest.

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