Regulatory mechanism of gene expression depending on the methanol concentration in the yeast used for heterologous protein production

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Research objective

Methanol-utilizing yeasts (methylotrophic yeasts) with strong methanol-induced gene promoters are widely used as hosts for heterologous protein production. The expression levels of methanol-induced genes change in response to the methanol concentration, but not in a concentration-dependent manner. The levels increase in the presence of 0.001–0.1% methanol, but decrease in the presence of methanol concentrations greater than 0.1%. We defined this phenomenon as "concentration-regulated methanol induction (CRMI)" and we are currently elucidating its molecular mechanism. Previously, we identified Wsc family proteins (KpWsc1/KpWsc3) as cell surface sensors that participate in the methanol-sensing mechanism in the methylotrophic yeast *Komagataella phaffii* (*Pichia pastoris*), and we reported that the deletion of both Wsc genes impairs CRMI.[1)] However, it is unclear how the methanol concentration information is transmitted to transcriptional regulators. To develop a heterologous protein-producing host that maximizes the potential of methylotrophic yeasts, we aimed to elucidate the molecular mechanisms underlying CRMI and its signal transduction pathway.

Methods

Among the transcriptional activators involved in methanol-induced gene expression, KpMxr1, which is a homolog of *Saccharomyces cerevisiae* Adr1, was suggested to be phosphorylated. Therefore, we analyzed the phosphorylation status of KpMxr1 at various methanol concentrations. We previously found that the serine residues of KpMxr1 were dephosphorylated under methanol culture conditions, and the threonine residues were phosphorylated in a methanol-concentration-dependent manner. In this study, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of KpMxr1 purified from cells cultured in the presence of glucose or methanol. We generated alanine-substituted mutants of serine and threonine residues that were predicted to be phosphorylated and analyzed their phosphorylation state and the *AOX1* expression level at various methanol concentrations.

To elucidate the signal transduction pathway to KpMxr1, constitutively active mutants of

the kinases KpPkc1 and KpMpk1, which function downstream of the Wsc family proteins, were expressed under the control of the Cu^{2+}-inducible *CUP1* promoter. We investigated *AOX1* expression levels and the phosphorylation status of KpMxr1.

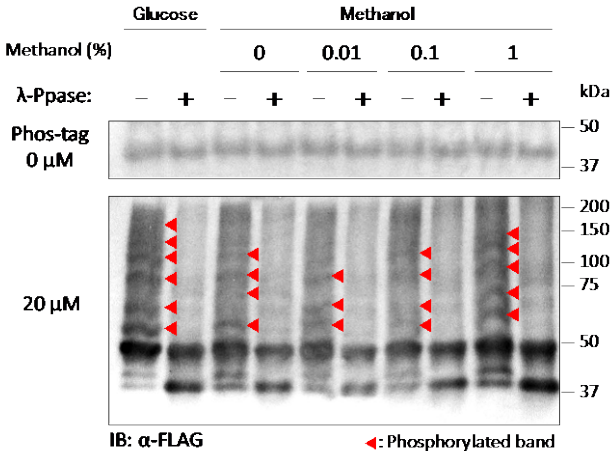

Fig. 1. Phosphorylation of KpMxr1$^{1\text{-}230}$-FLAG

Results

The LC-MS/MS analysis revealed that KpMxr1 had numerous phosphorylation sites. As most of the phosphorylated amino acid residues were in the 1–230 amino acid region, Phos-tag sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on the strain expressing KpMxr1$^{1\text{-}230}$-FLAG. We found that KpMxr1 was highly phosphorylated under glucose culture conditions and dephosphorylated under methanol culture conditions. We also found that the phosphorylation state of KpMxr1 changed with the methanol concentration (Fig. 1). Based on the LC-MS/MS results, the putative phosphorylated serine and threonine residues were replaced by alanine residues. We constructed strains expressing the KpMxr1 SA mutant (S110A/S111A) and TA mutant (T121A/T124A/T125A/ T128A/T131A). In these strains, the response to low concentrations of methanol decreased. In the TA mutant strain, the expression peak of *AOX1* shifted to a higher methanol concentration. These results revealed that strict control of the phosphorylation of serine and threonine residues in KpMxr1 is important for CRMI in *K. phaffii*.

Among the kinases in the cell wall integrity (CWI) signaling pathway, overexpression of KpPkc1^{R390P}, a constitutively active mutant of KpPkc1, decreased the *AOX1* transcript levels. However, overexpression of the constitutively active mutant KpMkk1^{S313P} did not affect *AOX1* transcript levels. Furthermore, the phosphorylation level of KpMxr1$^{1\text{-}230}$-FLAG and the transcript level of *AOX1* changed owing to the difference in the induction level of KpPkc1^{R390P} (Fig. 2).

Conclusion

Based on the results described above, the methanol concentration information from KpWsc1/KpWac3 is transmitted through an unknown pathway independent of the MAP kinase cascade that branches out from KpPkc1 to KpMxr1, and the phosphorylation status of KpMxr1 depends on the activation level of KpPkc1. Finally, we propose a CRMI pathway from Wsc family proteins to KpMxr1 (Fig. 3).[2)]

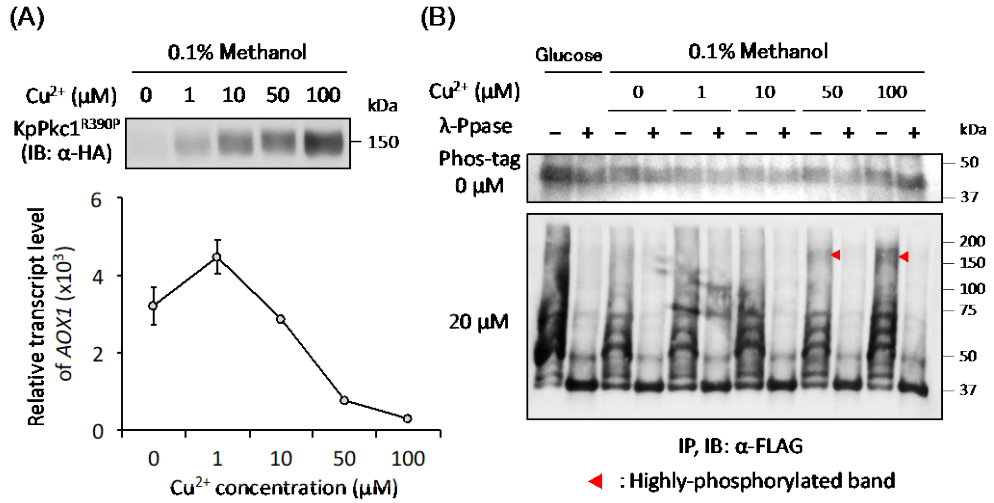

Fig. 2. Transcript level of *AOX1* (A) and phosphorylation of KpMxr1$^{1\text{-}230}$-FLAG (B) in cells expressing KpPkc1^{R390P}

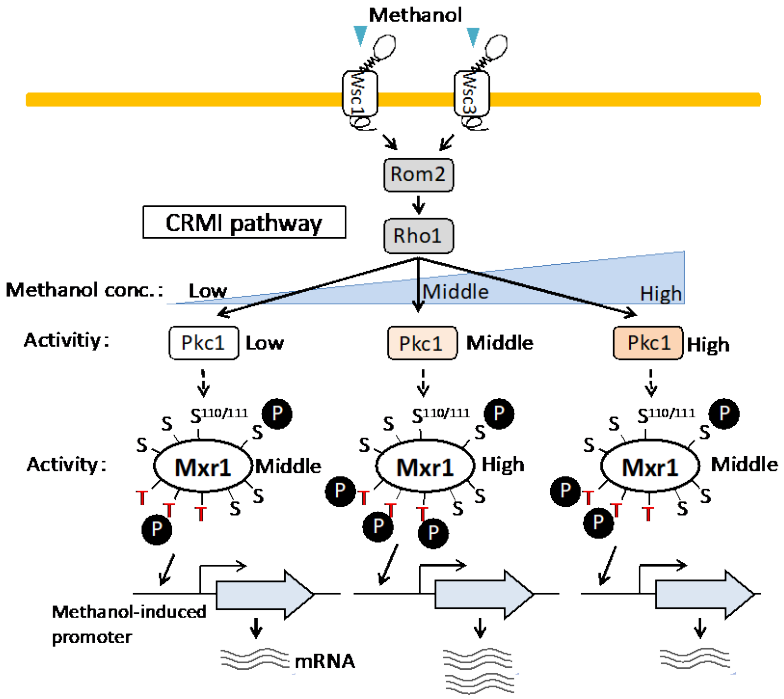

Fig. 3. Regulation of KpMxr1 phosphorylation by the CRMI pathway

References

1) Ohsawa, S., Yurimoto, H., and Sakai, Y. (2017) Novel function of Wsc proteins as a methanol-sensing machinery in the yeast *Pichia pastoris*. *Mol. Microbiol.* **104:** 349-363.

2) Inoue, K., Ohsawa, S., Ito, S., Yurimoto, H., and Sakai, Y. (2022) Phosphoregulation of the transcription factor Mxr1 plays a crucial role in the concentration-regulated methanol induction in *Komagataella phaffii*. *Mol. Microbiol.* **118:** 683-697.