Analysis of molecular mechanisms on nutrient source response in *Aspergillus oryzae*

Yujiro Higuchi
Faculty of Agriculture, Kyushu University

Research objective

The ability of cells to recognize and respond appropriately to nutrient sources is a fundamental mechanism in living organisms. The target of the rapamycin (TOR) complex is widely recognized as a molecular mechanism for nutrient source recognition in eukaryotes. The TOR complex (TORC) consists of TORC1 and TORC2, with TORC1 functioning primarily involved in nutrient source recognition and TORC2 functioning in the regulation of the actin cytoskeleton. Although studies on TORC1 have progressed in the budding yeast *Saccharomyces cerevisiae,* a model eukaryotic microorganism[1)], there is still no knowledge of TORC1 in the filamentous fungus *Aspergillus oryzae*. *A. oryzae* has been recognized as a 'national fungus' officially designated by the Brewing Society of Japan, and is a useful microorganism that has been employed in the fermentation and brewing industries in Japan for many years. However, the mechanisms of nutrient source response mechanisms remain largely unknown. In budding yeast, TORC1 is thought to localize primarily to the vacuolar membrane and trigger a nutrient source response. However, it has recently become clear that TORC1 functions in signaling endosomes in the vicinity of the vacuole[2)]. TORC1 activation promotes the biosynthesis of proteins, nucleic acids, and lipids, which are anabolic processes that inhibit autophagy, a catabolic process. Filamentous fungi, including *A. oryzae*, also form multinucleate and multicellular structures along elongated cell shapes called hyphae. However, little is known about when, where, and how mRNA is produced in such cells. Therefore, in this study, I aimed to understand the molecular mechanisms involved in nutrient source responses in *A. oryzae* cells, focusing on TORC1, which is believed to play a role in nutrient source responses, including amino acid and glucoamylase genes whose expression is regulated by sugar sources.

Methods

In budding yeast, Tor1p/Tor2p (paralogs), Kog1p, and Lst8p are present as TORC1 constitutive proteins. Similar to budding yeast, AoTor1 (AO090011000608), AoKog1 (AO090026000590), and AoLst8 (AO090012000914) are orthologs of TORC1 component proteins in *A. oryzae*, with 47%, 49%, 41%, and 6841% amino acid sequence homology, respectively. In the present study, we analyzed the function of TORC1 in *A. oryzae*. In budding yeasts, the genes encoding Tor1p/Tor2p and Kog1p are lethal. Therefore, conditional expression strains for both AoTor1 and AoKog1 were generated. Previously, conditional expression strain analyses were conducted for the functional analysis of lethal genes, where gene expression was suppressed by substituting the native promoter with the *nmtA*

promoter (*PnmtA*) in the presence of thiamine. Consequently, for the functional analysis of AoTor1 and AoKog1, strains were created in which the respective native promoters were replaced by P*nmtA* and phenotypic analysis was performed, focusing on growth comparisons.

The MS2 system was introduced to visualize *glaA* mRNA, which encodes glucoamylase, in live cells of *A. oryzae* by introducing 36 copies of the MBS sequence downstream of the *glaA* locus and expressing two copies of an enhanced green fluorescent protein (EGFP) fused to the MCP, which binds specifically to the MBS. Strains were also generated in which the red fluorescent protein mCherry was fused to the nuclear localization signal (NLS) and AoSec61 to label the nucleus and endoplasmic reticulum (ER), respectively. A THUNDER imaging system (Leica) was used for fluorescence microscopy analysis.

Results

1. Functional analysis of TORC1 components in *A. oryzae*

Conditional expression mutants were generated by replacing the endogenous promoter with the *nmtA* promoter in *Ator1* and *Aokog1*, and their growth was compared when the expression of each gene was suppressed in thiamine-supplemented medium. Unexpectedly, suppression of *Ator1* and *Aokog1* expression did not inhibit the growth of these mutants (Fig. 1A). When rapamycin, a known inhibitor of TORC1 in other organisms, was added, a lethal growth phenotype was observed when *Ator1* and *Aokog1* expression was suppressed (Fig. 1B). These results suggest that lethality occurred when rapamycin inhibited AoTor1 and AoKog1, which remained under the control of the *nmtA* promoter even in presence of the *nmtA* promoter.

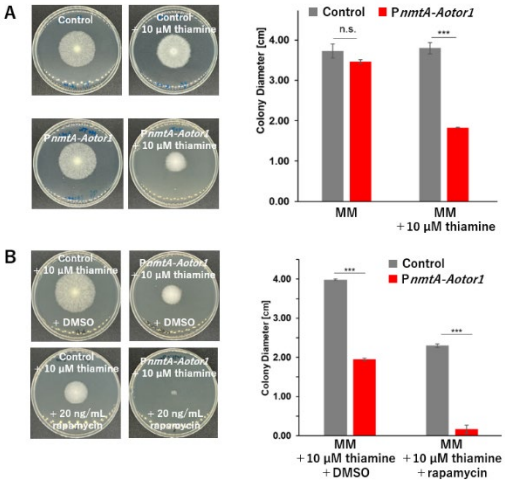

Fig. 1 Growth test of *Aotor1* conditional mutant

2. Analysis of expression dynamics of glucoamylase-encoding *glaA* mRNA

The strain in which *glaA* mRNA was visualized using the MS2 system (glaA-MS2 strain), was generated and analyzed using fluorescence microscopy. First, *glaA* mRNA was not detected in the cytoplasm in glucose-containing medium(Fig. 2). When transferred to maltose medium, dot-like fluorescence was observed in the cytoplasm (Fig. 2). This provides biological evidence that the transcription of *glaA* mRNA is induced in the presence of maltose. Further analysis of where in the mycelial cells *glaA* mRNA is produced revealed that it is generated in the nuclei of actively secreted areas, such as the hyphal tip and septum

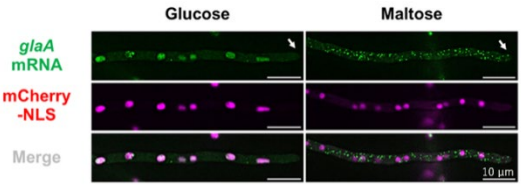

Fig. 2 Visualization of *glaA* mRNA
White arrow indicates the hyphal tip.

(Fig. 3).

Most *glaA* mRNAs were localized to the ER membrane, with some exhibiting kinesin motor-dependent dynamics (Fig. 4). It has been suggested that *glaA* mRNA is translated when it diffuses across ER membranes.

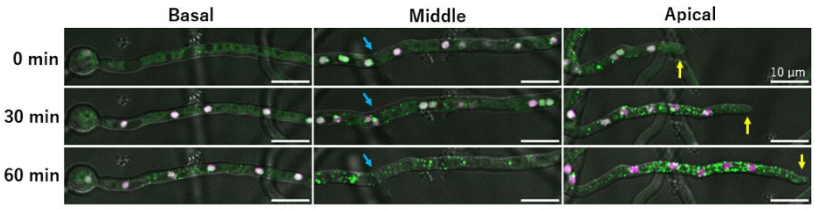

Fig. 3 Time-course of *glaA* mRNA generation
Yellow and blue arrows indicate the hyphal tip and septum, respectively.

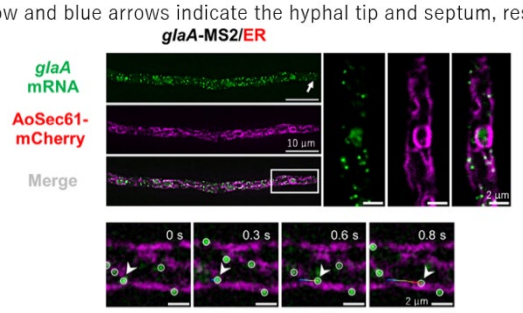

Fig. 4 Visualization of *glaA* mRNA and ER
White arrow and arrowheads indicate the hyphal tip and moving *glaA* mRNA, respectively.

Conclusion

Our study revealed one aspect of the mechanism of nutrient source response by TORC1-related factors in *A. oryzae*. Future studies should elucidate the molecular mechanisms of nutrient source responses mediated by AoTor1 and AoKog1. Additionally, a spatiotemporal regulatory mechanism of expression by changing the sugar source was revealed for the glucoamylase gene in *A. oryzae*. In the future, I would like to clarify other mRNAs and translational regulatory mechanisms to elucidate the molecular and physiological mechanisms of the nutrient source response in *A. oryzae* by clarifying other mRNAs and translational regulatory mechanisms, and link this to applied research, such as the production of useful materials.

References

1) Morozumi Y, Shiozaki K. (2021) Conserved and divergent mechanisms that control TORC1 in yeasts and mammals. *Genes* **12**: 88. doi: 10.3390/genes12010088.
2) Chen Z, Malia PC, Hatakeyama R, Nicastro R, Hu Z, Péli-Gulli MP, Gao J, Nishimura T, Eskes E, Stefan CJ, Winderickx J, Dengjel J, De Virgilio C, Ungermann C. (2021) TORC1 determines Fab1 lipid kinase function at signaling endosomes and vacuoles. *Curr Biol*. **31**: 297-309.e8. doi: 10.1016/j.cub.2020.10.026.
3) Morita Y, Takegawa K, Collins BM, Higuchi Y. (2024) Polarity-dependent expression and localization of secretory glucoamylase mRNA in filamentous fungal cells. *Microbiol Res*. **282**:127653. doi: 10.1016/j.micres.2024.127653.