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Outline of Research Result

The Pivotal Role of Membrane Traffic in the Growth and Differentiation of Filamentous Fungus, *Aspergillus oryzae*

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Research aims

Secretory and membrane proteins are transported in membrane-bound vesicles that bud from the donor organelle and fuse with the target membrane. In this process, SNARE proteins play pivotal roles in providing specificity and driving force for membrane fusion. In this study, we attempted to characterize SNARE proteins in *Aspergillus oryzae*, a filamentous fungus with a prominent ability to secrete proteins. In addition, possible involvement of vacuoles in the developmental process of filamentous fungi was proposed through in-depth analysis using AoVam3 protein, a vacuolar t-SNARE.

Methods

Amino acid sequences of 24 SNARE proteins in *Saccharomyces cerevisiae* obtained from the GenBank database were used to blast-search the *Aspergillus oryzae* genome database. The amino acid sequences of predicted *A. oryzae* proteins that showed significant similarity to yeast SNARE proteins and their open reading frame (ORF) sequences were extracted. 5′- and/or 3′-rapid amplification of cDNA ends (RACE) analyses were performed to determine the terminal position of cDNAs. The cDNAs encoding the putative SNAREs were amplified by PCR and used to construct expression plasmids that contain egfp (enhanced green fluorescent protein) -SNARE fusion genes under the control of *amyB* promoter and terminator, and have *niaD* selection marker. The plasmids were introduced into *A. oryzae* NS4 strain, and transformants were obtained. For microscopy, conidia of *A. oryzae* strains expressing fusion proteins were grown for 20 h.

Results

Identification of 21 genes encoding putative SNARE proteins in A. oryzae

We first searched the *A. oryzae* genome database for annotated open reading frames (ORFs) that encode proteins showing high similarity to SNARE proteins of *S. cerevisiae*. The search demonstrated the presence of 20 annotated ORFs except for Use1p ortholog. For some genes, 5′ and/or 3′ ends of the cDNAs were experimentally determined by RACE analysis. In addition, the genomic search identified a sequence that encodes a protein showing weak similarity to Use1p. Overall, the search demonstrated the presence of 21 SNARE-encoding genes in the *A. oryzae* genome.

Generation of strains that express fusion proteins of EGFP with SNAREs

We next generated strains expressing EGFP-SNARE proteins. We chose *amyB* promoter to drive the expression of fusion proteins. All the expression plasmids were constructed by MultiSite Gateway cloning system, using a 5′ entry clone containing *amyB* promoter, a center entry clone containing egfp, 3′ entry clones containing cDNAs encoding SNAREs, and a destination vector containing *amyB* terminator and *niaD* selection marker. The resultant plasmids were introduced into *A. oryzae* NS4 strain and at least four transformants were obtained.

Four putative SNAREs that localize to ER

A. oryzae has a set of SNARE orthologs in the transport of ER to Golgi; AoUfe1, AoSec20, AoUse1, and AoSec22. The fluorescence in the strains expressing EGFP-fused AoUfe1, AoSec20, AoUse1, and AoSec22 was primarily observed at ring-like structures, while a part of the fluorescence was observed at tubular or diffused structures in the cytoplasm. Staining with DAPI demonstrated that the fusion proteins were localized in the ring-like structures surrounding nuclei, suggesting that they are perinuclear ER. In addition, EGFP-AoSec20 and -AoUse1 resided in linear compartments near the plasma membrane (PM) and the septum, and were not stained with ER-Tracker. This result may reflect the presence of peripheral ER in filamentous fungi, and the preferential localization of AoSec20 and AoUse1 to this compartment.

Six putative SNAREs that localize to the Golgi apparatus

In strains expressing the either one of six EGFP-SNARE fusion proteins (EGFP-AoSed5, -AoBos1, -AoGos1, -AoBet1, -AoSft1, and -AoVti1), EGFP fluorescence showed similar distributions as punctate structures often found at the apical regions, or round structures (presumably vacuoles) in the proximal regions. In strains expressing EGFP-AoYkt6, EGFP fluorescence was present in the en-

tire cytoplasm and not restricted to any compartments; this seemed be due to mislocalization.

Four putative SNAREs that localize to the plasma membrane

The fusion proteins of AoSso1 and AoSso2 with EGFP localized to the plasma membrane and the septum, suggesting that these two putative SNAREs act in exocytosis. Also, AoSnc1 and AoNyv1 both localized to the plasma membrane, but the fluorescence of EGFP-AoNyv1 at the plasma membrane was far weaker than that of EGFP-AoSnc1. Therefore, AoNyv1 seemed to function elsewhere. In all strains in which EGFP fluorescence was observed at the plasma membrane, the fluorescence evenly covered the cell surface except that it tended to be weak at hyphal tips, and no particular tip-high gradient of the fluorescence was observed. In strains expressing EGFP-AoSec9, the fluorescence occupied the entire cytoplasm, as in the strains expressing EGFP-AoYkt6. We speculated that the fusion with EGFP might have inhibited the anchorage to the plasma membrane of AoSec9.

Three putative SNAREs that localize to vacuolar membrane

Vacuolar localization of AoVam3 in *A. oryzae* has been reported. As AoVam3, EGFP-AoVti1 and -AoVam7 localized to the membrane of vacuoles that were stained by CMAC. EGFP-AoNyv1 also localized to the vacuolar membrane, suggesting that AoNyv1 has dual roles as exocytic and vacuolar R-SNAREs.

Putative SNAREs that localize to endosomes

In filamentous fungi, early endosomes are thought to be the compartments that show rapid (5 μm/sec) movement along the hyphal axis, while late endosomes are thought to be static, adjacent to vacuoles and can be stained by FM4-64. The fusion proteins of five SNARE orthologs, AoTlg1, AoTlg2, AoVti1, AoSyn8, and AoSnc1 localized to mobile particulate compartments. The compartments were stained by FM4-64. The fusion proteins of five SNAREs, AoTlg2, AoVti1, AoNyv1, AoVam7, and AoSyn8 localized to static compartments. The former and latter structures were suggested to be early and late endosomes, respectively.

Putative SNAREs that localize to the septum

Some of the fusion proteins of SNAREs localized at/near the septum. These include three groups of SNAREs; the plasma membrane SNAREs AoSso1, AoSso2, and AoSnc1; ER SNAREs AoSec20 and AoUse1; and endosomal/vacuolar SNAREs AoTlg1, AoVti1, and AoNyv1. EGFP fluorescence of the fusion proteins of the plasma membrane SNAREs most likely reflects the septal membrane. In strains expressing the fusion proteins of ER SNAREs, EGFP fluorescence seemed to be present as doublet on both sides of the septum of two adjacent compartments.

A novel role of vacuoles in filamentous fungi

Next we analyzed the localization of AoVam3-EGFP in more detail. In young hyphae AoVam3-EGFP localized on the vacuolar membrane, while in the old hyphae cultured for several days the EGFP fluorescence was distributed into the vacuolar lumen. Since the EGFP moiety of the fusion protein resides on the cytoplasmic side of the vacuoles, this topological change of EGFP fluorescence imply that the vacuolar membrane invaginated into the lumen, presumably by budding into the luminal side of the vacuole. To examine the possibility that in the old hyphae other cytoplasmic contents are similarly transported into the vacuole, we observed the localization of nuclei, mitochondria, and peroxisome; these organelles were visualized by the expression of EGFP-fusion proteins that specifically localize to the corresponding organelles. As expected, the EGFP fluorescence was exclusively observed in each organelle in young hyphae. In contrast, the fluorescence was also observed in the vacuoles in old hyphae. Moreover, this shift in localization did not occur in the strain deleted for *Aoatg8*, a gene essential for autophagy. Taken together, these results suggest that vacuoles in the old hyphae of filamentous fungi take up and degrade cytoplasmic organelles via autophagy, presumably for the purpose of recycling cellular components.

Conclusion

In summary, the localization and possibly function of the ER, Golgi, and endosomal SNAREs seem to be largely conserved between *S. cerevisiae* and *A. oryzae*. It is thus likely that the fluorescence of EGFP-SNARE reflects the spatial distribution of each organelle where the SNARE protein localizes. Some SNAREs localized to more than one cellular compartments. Intriguingly, the analysis using AoVam3 demonstrated that the fungal vacuoles in the old hyphae take up various organelles. Since this process was dependent on the presence of the autophagy gene, it is suggested that the vacuoles in filamentous fungi play pivotal roles in degrading unnecessary intracellular organelles in the old hyphae and recycling cellular components to young, actively-growing hyphae.

References

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