論 文 の 内 容 の 要 旨

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論 文 題 目　**Transcriptional and cytological studies on the regional differences of *Aspergillus oryzae* mycelia**
(麹菌 ***Aspergillus oryzae*** の菌糸部位特異的発現制御に関する研究)

The filamentous fungus *Aspergillus oryzae* has been utilized in the process of traditional fermented food and beverage production to breakdown macromolecules in the substrates. Its exceptional ability to secrete a variety of hydrolases in large amounts and the safety status of the organism for human consumption have captivated the attention of enzyme production and other biotechnology industries to employ *A. oryzae* as a host for homologous and heterologous protein production.

When filamentous fungi are grown on solid substrates, morphologically distinct regions are formed, such as hyphae growing on the surface of the substrate, those growing into the substrate, aerial hyphae, and conidiophores. One cell type can be derived from a different cell type, and the ability to adopt any cell type enables the organism to adapt to many environments or conditions, and to colonize the substrate extensively. The different cell types are presumed to be functionally distinct; many hydrolytic enzymes are secreted from tip cells, hyphae growing into the substrate are thought to provide nutrients to other parts of the mycelium, and some aerial hyphae differentiate into conidiophores to generate conidia. The details, for example, on what distinguishes these cells have not been studied.

The objective of this study is to demonstrate the differences that exist between the three regions in the mycelium of *A. oryzae* and to discover gene(s) responsible for the regulation, maintenance, and/or differentiation of the regions. In addition, a novel method was developed that allowed the extraction of biological samples from three morphologically distinct regions of the mycelium.

I Visualization of the distribution of the secreted protein RntA in hyphae of *A. oryzae*

Extracellular hydrolytic enzymes in filamentous fungi secreted from the tip cells of the hyphae are modified and transported by the secretory pathway. Due to the unique morphology of filamentous fungi, the distribution of the organelles and the components of the secretory pathway may not be similar to those of other eukaryotes. To visualize the location and the distribution patterns of secreted proteins within the hyphae of *A. oryzae*, a fusion protein was constructed with the secreted protein RNase T1 (RntA) and the green fluorescent protein EGFP. The fusion protein, RntA-EGFP, was distributed evenly throughout the tip cells with the highest fluorescence in the apical region of the tip cells. On the contrary, RntA-EGFP aggregated in unidentified cytoplasmic structures in various parts of the hyphal compartments.

The distribution of RntA-EGFP in the tip cells was further examined to visualize the intermediate distribution patterns of the secretory pathway under conditions of stress. Changes in growth temperature caused RntA-EGFP to localize in the vacuoles. In addition, the distribution patterns of RntA-EGFP fluorescence in the presence of protein transport inhibitors (brefeldin A, cytochalasin A, and nocodazole) differed between treatments. The addition of these inhibitors caused RntA-EGFP to relocalize in structures analogous to the ER and the Golgi apparatus, at the cell surfaces of hyphae, and in unrecognized structures. In conclusion, the study of RntA-EGFP under conditions of stress allowed the visualization of the distribution of the intermediate organelles in the secretory pathway and of the fate of the secreted protein.

II Development of the novel culture method – The square-plate culture method

The observation of the distinct distribution patterns of RntA-EGFP between the tip cells and the non-tip cells suggested that the two types of cells have characteristic functions. To analyze the components or the processes that cause this difference, a novel culture method, the square-plate culture method, was developed. The idea for the method derived from the race tube of *Neurospora crassa*, in which the fungus is inoculated on one end of a test tube to constrain its growth to one direction towards the other end. Concurrently, when *A. oryzae* wild type RIB40 was plated on different nutrient agar media, three morphologically distinct regions were detected in mycelia cultured on rich media. The edge of the mycelium contained transparent tip cells on the surface of the medium ("tip" region). Behind these cells, white-colored aerial hyphae were observed ("white" region). The furthest region from the edge of the mycelium was comprised of aerial hyphae with conidia and the region was colored due to the pigmentation of conidia ("basal" region). The characteristics of the mycelium grown by the square-plate culture method were identical to that on round plates, except that the regions were horizontally aligned in a band-like pattern. The three morphologically distinct regions were cut out from the mycelium, and the extraction of biological samples such as RNA, proteins and DNA from each of the regions became possible.

III Differential expression of genes and localization of proteins in the mycelium

Differences between the three regions were investigated from diverse angles, and the analyses revealed that these were not restricted to the visible macroscopic features, but were also observed at the molecular and genetic levels. The secretion of α-amylase was detected only at the "tip" region of the mycelium by Western blot analysis of the mycelia. Furthermore, differences were observed in the morphology, size, and number of organelles when mycelia growing on solid agar medium were analyzed under the microscope. Vacuoles were tubular at the "tip" region and gradually increased in size until they filled most of the areas of the compartmentalized cells in the "basal" region. Moreover, the number of nuclei decreased dramatically in the "basal" region. Functional differences, such as secretion, and changes in morphology, size, and number of organelles across the mycelia implied that a regulation at the genetic level exists, and this regulatory mechanism may be controlling the expression of genes and proteins that play a role in defining the regional features.

The differential expression of known genes of *A. oryzae* was analyzed next by reverse-transcriptase PCR (RT-PCR) to verify the hypothesis described above. Secreted proteins α-amylase and glucoamylase showed a gradient-like change in expression with the highest at the "tip" region. The conidial hydrophobin, *rolA/hypA*, known to be expressed in the conidia, was only detected in the "basal" region. Based on these preliminary analyses, it was demonstrated that the mycelia was appropriately divided into three regions and that a gene or group of genes have a characteristic expression pattern across the mycelia.

IV Screening of genes expressed in morphologically distinct regions of the mycelium by microarray analyses

After the differential expression of the selected *A. oryzae* genes was confirmed by RT-PCR, novel genes expressed specifically in each of the three regions were screened using the cDNA microarray chip NRIB3000. Two microarray experiments were carried out in this study. One compared the "tip" region with the "white" region and the second compared the "basal" region with the combination of the "tip" and "white" regions. The genes expressed at high level (ratio value of > 2.5) were grouped into their respective Clusters of Orthologous Groups (COG) of proteins, and regional characteristics were recognized. Many of the genes that were expressed at higher levels in the "tip" region belonged to the category of "translation, ribosomal structure and biogenesis", indicating that the expression of genes involved in protein synthesis was up-regulated. A functional category specific to the "white" region could not be determined; nevertheless, RT-PCR analysis confirmed that the "white" region was a region where "tip" region-specific genes were down-regulated and "basal" region-specific genes were up-regulated. In the list of genes expressed at high levels in the "basal" region, many genes coded for macromolecule transporters/permeases. In conclusion, the screening method identified previously unknown genes to be specifically expressed in a region of a mycelium. Moreover, the detection of a predominant functional group(s) in a region indicated that the predominant function(s) may be governed by the genes expressed in that region – an outcome expected, but never previously demonstrated empirically.

V Characterization of genes expressed at high levels in the "tip", "white", or "basal" regions of the mycelium

The advantage of using the reverse genetics approach to study an organism is that the putative function of the genes could be predicted *in silico* to some extent. To further study the influence of the genes expressed at high levels on the characteristics of a region, three genes from the "tip" region (magnesium-dependent phosphatase, Rho GTPase activating protein, transcriptional activator p100) and two from the "white" region (*A. oryzae* hydrophobic surface binding protein (*hsbA*) and an unknown protein) were selected. The predicted functions were determined, and gene deletions were carried out using the *ku70* deleted strain, which allows homologous recombination to occur and acquire the deletion mutants at a high rate in *A. oryzae*. Deletion mutants were obtained, but distinguishable phenotypic differences compared to the parental strain were not detected during growth on rich or minimal media. The current results from the cumulative deletion analysis suggest that the genes chosen were not directly involved in the fundamental regulation of hyphal growth and development, and gene specific analyses are required to determine their role in the region.

Conclusion

This research aimed towards screening and discovering new genes involved in the high secretion ability of the filamentous fungus *A. oryzae* by determining and understanding the differences between the "tip" cells that have the ability to secrete and the other cell types that cannot. The culture method developed for the purpose of extracting biological samples from the three morphologically distinct regions of the fungal mycelium successfully extracted RNA from the regions and allowed to study the differences between them. Moreover, this study empirically demonstrated the existence of up- and down-regulation of gene expression within a mycelium of filamentous fungi, which was only hypothesized by many fungal researchers. The differences in the contents of gene transcripts in a certain region of the mycelium may be influencing the characteristics of that region, observed macro- and microscopically. By further analyzing the genes identified in this study, it is hoped that a deeper understanding of the features of each region can be achieved.

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