博士学位論文　主論文の要約

論文題目

Nutrient-dependent predominant secretion of GH family enzymes by *Aspergillus* spp isolated from fields in subtropical region

（亜熱帯のフィールドから分離したアスペルギルス属糸状菌による GH ファミリー酵素群の栄養依存的優勢分泌）

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containing protein-encoding gene

- FG-GAP repeat protein secretion was regulated independently of the secretion of cellulose- and hemicellulose-degrading enzymes
- Swollen cellulose enhanced secretion of FG-GAP repeat protein and beta-1,3-glucan hydrolyzing enzymes (own cell wall remodeling enzymes: endo- and exo-beta-1,3-glucanases and de-branching endo-beta-1,3-glucosidases) by *Aspergillus fumigatus* UY015
- *Aspergillus oryzae* RIB49 secreted FG-GAP repeat protein dominantly comparable to *Aspergillus fumigatus* UY015
- FG-GAP repeat protein gene might be present only *Aspergillus* sections *Flavi* and *Fumigati* in genus *Aspergillus* and percent identity in amino acid sequence homology was discontinuous with distinct gap between the two *Aspergillus* sections
- Nucleotide sequences in the UTR and flanking regions of FG-GAP repeat protein gene were distinct between the sections *Fumigati* and *Flavi*, and also among three clades in *Aspergillus* section *Flavi*

要約

The most abundant form of cellulose in nature is a linear and mostly crystalline microfibril with a 10-nm diameter, termed type I cellulose, consisting of 36 paracrystalline cellulose chains with lengths of 2,000-2,5000 glucose residues. Due to their paracrystalline nature, cellulose microfibrils are insoluble in aqueous solution and even impermeable to water and, therefore, highly resistant to enzymatic hydrolysis. Thus, cellulose is unable to be assimilated by microbes without cellulose breakdown systems and of course by humans and animals, lacking genes for cellulose hydrolysis enzymes, without help of intestinal commensal microbes. In nature, at dead plant tissues, various microbes including cellulolytic filamentous fungi, actinomycete bacteria and anaerobic bacteria degrade cellulose constituting plant cell wall. Resultant sugar products such as glucose and cellooligosaccharides (cellodextrin) are taken up as nutrients by themselves and in part by other microbes that are unable to degrade cellulose. Extended hyphae of cellulolytic filamentous fungi are able to penetrate cellulose exposed on plant tissues and, thereby, cellulolytic enzymes secreted locally and degrade cellulose effectively and synergistically at the sites of fungal penetration. By contrast to cellulolytic fungi, anaerobic bacteria including clostridia and ruminal bacteria are unable to penetrate cellulose substrates. Instead, such bacteria form protein complexes, called cellulosome, constituting high molecular-mass proteins with cellulose-binding domain in addition to cellulose-degrading enzymes. These locally acting enzyme systems of cellulolytic filamentous fungi and bacteria on the surface of solid cellulosic substrates might enable effective degradation of cellulose substrates by the gradual and synergistic enzymatic reactions and the preferential access to cellulose degradation products in competitive environment.

Biological degradation of cellulose from dead plants in nature and plant biomass from agricultural and food-industry waste is important for sustainable carbon recirculation. This study aimed at searching diverse cellulose-degrading systems of wild filamentous fungi and obtaining fungal lines useful for cellooligosaccharide production from agro-industrial wastes. Fungal lines with cellulolytic activity were screened and isolated from stacked rice straw and soil in subtropical fields. Among the isolated lines, in liquid culture with a nutrition-limited cellulose-containing medium, four lines of *Aspergillus* spp secreted 50~60 kDa proteins as markedly dominant components and gave clear activity bands of possible endo-β-1,4-glucanase in zymography. Mass spectroscopy (MS) analysis of the dominant components identified three endo-β-1,4-glucanases (Glycoside Hydrolase family: GH5, GH7 and GH12) and two cellobiohydrolases (GH6 and GH7). Cellulose degradation by the secreted proteins was analyzed by LC-MS-based measurement of derivatized reducing sugars. The enzymes from the four *Aspergillus* spp produced cellobiose from crystalline cellulose and cellotriose at a low level compared with cellobiose. Moreover, though smaller than that from crystalline cellulose, the enzymes of two representative lines degraded powdered rice straw and produced cellobiose. These fungal lines and enzymes would be effective for production of cellooligosaccharides as cellulose degradation-intermediates with added value other than glucose.

Under a standard liquid culture condition in nutrient-rich medium containing crystalline cellulose as a sole carbon source, by contrast to dominating secretion of cellulose degrading enzymes under nitrogen-poor condition, several wild lines of *Aspergillus* spp dominantly secreted a protein of about 30 kDa, chemically identified to be a fungal secreted protein, termed FG-GAP repeat protein, similar to a mammalian cell-adhesion molecule, integrin alpha-subunit. A wild line *Aspergillus fumigatus* UY015 began to secrete the protein a few days after a start of the culture and prior to the secretion of cellulolytic enzymes, whereas a standard Aspergillus *nidulans* A4 did not at all. Another standard *Aspergillus oryzae* RIB40 secreted it in a manner similar to *Aspergillus fumigatus* UY015. *Aspergillus fumigatus* UY015, and *Aspergillus nidulans* A4 as well, secreted endo-1,3-beta-glucanase (eglC), which hydrolyzes fungal cell wall beta-glucan, rather than cellulolytic endo-1,4-beta-glucanses at a protein level. Swelling of crystalline cellulose markedly enhanced the secretion of ~60 kDa-high molecular isoform of FG-GAP repeat protein, catalase and eglC. Hemicellulose (xylan) induced weak or almost no secretion of FG-GAP repeat protein to the wild lines A. fumigatus UY015 and A. oryzae/flavus UY019, despite that both of cellulolytic and xylanolytic activity were induced remarkably. By contrast, a natural substrate, rice straw, strongly induced secretion of FG-GAP repeat protein, both of cellulolytic and xylanolytic activity, and 1,4-beta-glucan cellobiohydrolase B. BLAST search of protein sequence indicated that FG-GAP repeat protein gene is present only in a limited number of distantly related *Aspergillus* species, including *Aspergillus fumugatus* and *Aspergillus oryzae*, in *Aspergillus* sections *Fumigati* and *Flavi*. Moreover, genomic DNA sequences around the gene showed that *Aspergillus fumigatus* Af973 and UY015 have 5'-flanking sequences distinct

from *Aspergillus oryzae* RIB40 and UY017. Phylogenetic analysis of the coding and flanking sequences of FG-GAP repeat protein gene indicated that there is clear distinction in these nucleotide sequences not only inter-sectionally between the section *Flavi* and the section *Fmigati* but also intra-sectionally within *Aspergillus* section *Flavi*. Taken these together, it is suggested that cellulose substrate alone first induces secretion of FG-GAP repeat protein and endo-1,3-beta-glucanase and additional hemicellulose substrate induces endo-1,4-glucanse and cellobiohydrolase, and that FG-GAP repeat protein genes in *Aspergillus fumugatus* and *Aspergillus oryzae* genomes originated evolutionarily from distinct ancestral genes and evolved adaptably as a gene involved in fungal cell growth.

Blast homology search analysis on FG-GAP-repeat protein genes using fungal whole genome sequence databases, including those of 20 *Aspergillus* spp, suggested that FG-GAP-repeat protein genes, at least in fungi, is markedly different phylogenetically from general house-keeping genes, including rRNA-encoding genes and metabolic enzyme genes. In a recent study on phylogenetic analysis of Aspergillus spp using their whole genome sequences, all of *Aspergillus* spp belonging to section Flavi located in a single clade and clearly separated from the other *Aspergillus* spp, such as *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus nidlans*, and *Aspergillus niger*. In the present study, the phylogenetic trees based on CDS sequences, in both amino acid and nucleotide levels, and rRNA-encoding gene D1/D2 region showed a tree diagram similar to that of whole genome sequence, i.e., fungal species and strains in section *Flavi* located in a single thick clade. On the other hand, when phylogenetic trees were made based on the 5'- and 3-prime flanking sequences, fungal species and strains in section *Flavi* distributed separately in multiple clades. Such difference in tree diagram, i.e., sequence homology, between CDS and flanking region of FG-GAP-repeat protein gene in addition to the presence of FG-GAP-repeat protein gene only in a few sections of genera *Aspergillus* suggest that FG-GAP repeat protein gene had been acquired during fungal evolution in a way different from general house-keeping genes. To elucidate biological roles of FG-GAP repeat protein in plant cell-wall degradation for fungal growth, further studies on genomic structures and protein functions are required of not only FG-GAP repeat protein, but also the other homologous proteins, such as integrin alpha N-terminal containing protein and ulilysin of filamentous fungi.