論文の内容の要旨

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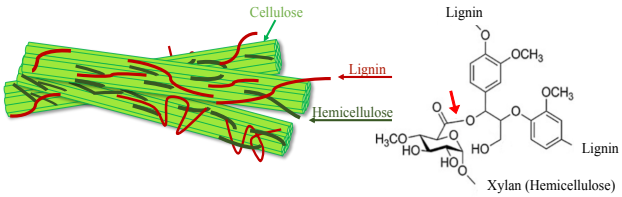
論文題目

Analysis on physiological function of glucuronoyl esterase in plant biomass utilization of *Neurospora crassa*
（アカパンカビの植物バイオマス利用におけるグルクロン酸エステラーゼの生理機能の解析）

Introduction

Plants, through photosynthesis, store solar energy in the form of cell wall polymers such as cellulose, hemicellulose, and lignin. Although biomass material is cheap and easy to obtain, the relatively high cost of converting biomass to fermentable sugars remains the primary obstacle in using plant biomass to benefit mankind and promote the development of society. In nature, filamentous fungi act as critical degraders of plant biomass and play an important role in the carbon cycle. The model filamentous fungus *Neurospora crassa* is known to have the ability to utilize plant biomass (e.g. grass, wood, and straw) as the sole carbon source to maintain its growth, indicating that it has a strong ability to convert plant biomass to fermentable sugars. Studies have shown that this fungus upregulates the expression of a variety of genes encoding hydrolytic enzymes, especially cellulases and hemicellulases, involved in the degradation of lignocellulose when grown on plant biomass. This gene expression regulatory mechanism in *N. crassa* includes several key transcription factors, such as CLR-1 (NCU07705), CLR-2 (NCU08042), and XLR-1 (NCU06971), which are activated or repressed by specific fermentable sugars.



Glucuronoyl esterases (GEs) target site is indicated by the red arrow.

Besides cellulases and hemicellulases, a family of relatively newly-discovered enzymes, glucuronoyl esterases (GEs) that are classified into the Carbohydrate Esterases family 15 (CE15), are considered as promising biological tools for the degradation of plant biomass since they disconnect the ester linkage between 4-*O*-methyl-D-glucuronic acid residues of glucuronoxylans and aromatic alcohols of lignin, thus loosening the rigid structure of lignocellulose. Interestingly, although GE itself does not contribute to the release of reducing sugars, a boosting effect of GE on the degradation of plant biomass by GH10 xylanase was observed. Currently, studies of GEs primarily focus on their functions *in*

vitro even though more than a decade has passed since its discovery from *Schizophyllum commune* in 2006, limiting our understanding of their physiological roles and potential application.

To gain insights into the physiological functions of GEs in the deconstruction and utilization of wood polymers *in vivo*, in this study *N. crassa* was used as a model organism. First, the *in vitro* experiments were carried out to confirm that a GE in *N. crassa*, *Nc*GE (NCU09445), produced in *Pichia pastoris* facilitated the deconstruction of plant biomass by mixed cellulases or xylanase. Then *N. crassa* wild type (WT), *Nc*GE-overexpression (*NcGE*^{oex}), and the catalytically inactive-*Nc*GE-overexpression (*Mut-NcGE*^{oex}) strains were generated and used to evaluate the effects of *Nc*GE in the utilization of wood materials during growth. Additionally, deletion of the major transcription factor genes, *clr-1*, *clr-2*, and *xlr-1*, individually and in combination, was conducted to explore the pathway through which *Nc*GE induces the expression hydrolytic enzyme genes. Furthermore, the physiological function of xylanolytic enzyme-encoding genes in *N. crassa* was analyzed to elucidate their roles in the process of plant biomass deconstruction and utilization.

Chapter 1. Analysis of synergistic effects of *Nc*GE and plant biomass-degrading enzymes

The previous study conducted in this laboratory had confirmed that the recombinant *Nc*GE produced in *P. pastoris* was capable of cleaving the linkage of 4-*O*-methyl-D-glucuronic acid bond of the synthetic substrate analog of glucuronic acid, benzyl methyl α-D-glucopyranosiduronate. In this study, the natural substrates, mulberry wood powder and Lignin Rich Precipitate (LRP) were used to examine the synergistic effects of *Nc*GE with the commercial cellulase, cellobiohydrolase (CBH), and xylanase preparations in the degradation of plant biomass *in vitro*. Results showed that when *Nc*GE acted together with cellulase or xylanase on the substrates, the release of reducing sugars was significantly enhanced compared to when cellulase or xylanase was used alone, suggesting that *Nc*GE can stimulate biomass degradation.

The catalytically inactive mutant of *Nc*GE (Mut-*Nc*GE) was designed by mutating Ser at the catalytic center to Ala. For the purpose of deciphering the physiological function of GE *in vivo*, both *NcGE* and *Mut-NcGE* tagged with c-myc and 6×His tags, whose expression was driven by a strong constitutive promoter P*ccg-1*, were transformed to the *N. crassa* WT strain. The significantly higher GE activity in the culture supernatant of *NcGE*^{oex} strain, but not of *Mut-NcGE* strain, along with the result of Western blot confirmed the successful construction of overexpression strains. qRT-PCR analysis showed that the expression level of the transcription factors *clr-2* and *xlr-1* was higher in *NcGE*^{oex} strain compared to those in WT and *Mut-NcGE*^{oex} strains when they were grown in mulberry wood extract medium. In line with this, two cellulase genes *cbh-1* and *gh5-1* encoding CBH and endoglucanase, respectively, as well as three xylanolytic enzyme genes, *gh10-1*, *gh10-2*, and *gh3-7*, were dramatically upregulated in *NcGE*^{oex} strain, but not in WT and *Mut-NcGE*^{oex} strains. Furthermore, compared to WT and *Mut-NcGE*^{oex} strains, a consistently higher secreted xylanase activity was observed in *NcGE*^{oex} strain when they were grown in the media containing different kinds of wood

materials (mulberry wood, pinewood, and beechwood, respectively) as the sole carbon source. These results suggest that the overproduction of *Nc*GE led to increased expression of transcription factors, which then caused enhanced transcription of major hydrolytic enzyme genes and activity of secreted xylanase. Therefore, *Nc*GE was shown to boost not only the degradation of plant biomass *in vitro* but also the expression of genes encoding biomass-degrading enzymes *in vivo.*

Chapter 2. Elucidation of *Nc*GE-involved biomass degradation mechanism in *N. crassa*

In *N. crassa*, CLR-1 and CLR-2 are two essential transcription factors responsible for the vast majority of cellulolytic responses, while XLR-1 mainly plays a role in the xylanolytic response. In order to explore the pathway through which *Nc*GE induces gene expression, strains deleted for *clr-1*, *clr-2*, and *xlr-1*, in WT and *NcGE*^{oex} backgrounds, were constructed. Deletion strains for *clr-1* and *clr-2* (Δ*clr-1*, Δ*clr-2*, *NcGE*^{oex}-Δ*clr-1*, and *NcGE*^{oex}-Δ*clr-2*) showed significantly reduced transcript levels of a set of genes encoding the hydrolytic enzymes and secreted enzyme activity compared to WT strain. Deletion of *xlr-1* mainly downregulated the expression of xylanolytic enzyme genes, and overexpression of *Nc*GE partially restored the transcription of *clr-2*, *cbh-1*, *gh5-1*, and the extracellular β-xylosidase genes (*gh3-7* and *gh3-8*); the activity assay of secreted enzymes confirmed the expression profile of these strains. Considering the fact that transcription factors modulate plant biomass degrading enzymes upon soluble sugars, I speculated that the enhanced expression of hydrolytic enzymes in the *NcGE*^{oex} strain might be due to the generation of hypothetical inducer(s) released from the wood substrate by the action of GE that in turn activates the intracellular transcription factors.

To examine the presence of hypothetical inducer(s), the effects of hydrolysates prepared by treating mulberry wood with *Nc*GE- and/or xylanase in the gene expression were examined. qRT-PCR results demonstrated that the expression of *NcGE, gh3-4*, *gh10-4*, *gh11-1*, *gh3-7*, *gh43-1*, and *gh43-5* was highly upregulated by *Nc*GE-treated biomass hydrolysate, whereas *xlr-1*, *cbh-1*, *gh10-2*, and *gh11-2* showed elevated transcription in response to *Nc*GE plus xylanase-treated biomass hydrolysate. These results suggest that the hydrolysates from *Nc*GE- and *Nc*GE plus xylanase-treated biomass contained elements that triggered the expression of hydrolytic enzymes. Taken together, the mechanism whereby *Nc*GE regulates the expression of hydrolytic enzymes is proposed as follows: secreted *Nc*GE disconnects the linkage between xylan and lignin, which facilitates the degradation of xylan by xylanases. This leads to the loosening of wood matrix and makes plant biomass-degrading enzymes more accessible to the substrates. Subsequently, unknown inducer(s) such as oligosaccharides are released and incorporated, which then activate the transcription factors *clr-1*, *clr-2*, and *xlr-1*. Finally, the expression of hydrolytic enzymes was boosted.

Chapter 3. Investigation of xylan degradation and utilization system in *N. crassa*

Considering that *Nc*GE facilitates biomass degradation by xylanases, the biochemical and physiological properties of xylanolytic enzymes in *N. crassa* were investigated to illustrate their roles in biomass deconstruction. By analyzing the transcriptome data, I identified six

xylanases (GH10-1, GH10-2, GH10-3, GH10-4, GH11-1, and GH11-2) and four β-xylosidases (GH3-7, GH3-8, GH43-1, and GH43-5) that were predicted to be involved in xylan utilization. qRT-PCR results showed upregulation of these genes when the cells were grown in xylan medium. First, the subcellular localization of these proteins was analyzed by expressing EGFP-fusion proteins. As a result, except for two putative intracellular xylosidases, GH43-1 and GH43-5, which were found to be distributed in the cytoplasm, the other eight proteins localized at the septal area and cellular membrane or tips, indicating that they were secreted. Next, the enzymatic characterization was performed. Expect for three previously-characterized β-xylosidases (GH3-7, GH43-1, and GH43-5), the expression of remaining seven genes were attempted, of which four xylanases, GH10-1, GH10-2, GH11-1, and GH11-2, were successfully produced in *E. coli* and purified. When xylotetraose (X4) and xylooligosaccharides (XOS) with a higher degree of polymerization (DP) were used as the substrates, GH10-1, GH10-2, and GH11-2 released xylobiose as the minimum hydrolytic product; in contrast, the minimum cutting unit of GH11-1 was xylotriose and it only hydrolyzed XOS with a DP larger than four.

To clarify the *in vivo* roles of these genes, the growth of ten single xylanolytic enzyme gene deletion strains together with two double intracellular and extracellular β-xylosidase deletion strains (Δ*gh43-1*Δ*gh43-5* and Δ*gh3-7*Δ*gh3-8*, respectively) was investigated. In the sucrose medium, except for a slightly reduced growth of Δ*gh43-1*Δ*gh43-5* strain, others showed growth similar to WT. However, when grown in xylan or mulberry wood extract media, Δ*gh10-1*, Δ*gh10-2*, Δ*gh3-7*, and Δ*gh3-7*Δ*gh3-8* exhibited reduced growth compared to WT, but the deletion of *gh43-1* (Δ*gh43-1* and Δ*gh43-1*Δ*gh43-5*) showed even severer growth defect compared to others. Additionally, the intracellular β-xylosidase activity almost vanished in Δ*gh43-1*, indicating the critical role of GH43-1 in xylan utilization. Collectively, these data indicate that in *N. crassa* the secreted xylanases GH10-1, GH10-2, and β-xylosidase GH3-7 play roles in hydrolyzing the surrounding biomass substrates, while GH43-1 is indispensable for XOSs utilization inside the cell.

Conclusion

This study provided both *in vitro* and *in vivo* evidence on how *Nc*GE boosts the degradation of plant biomass. Especially, it was proposed that the induction of genes involved in biomass degradation was due to the release of inducer(s) from the biomass substrate through the cooperative action of *Nc*GE and hydrolytic enzymes. Functional characterization and physiological analysis of xylanolytic enzymes have given a better understanding on the mechanism of plant biomass deconstruction in *N. crassa*. Additional efforts toward clarifying the identity of the hypothetical inducer(s) and the physiological crosstalk between *Nc*GE and hydrolytic enzymes *in vivo* as well as defining the functions of GH3-8, GH10-3, and GH10-4 will provide a more profound insight into the rational design of industrial strains through precise tailoring of metabolic networks to achieve the goal of complete deconstruction of raw plant biomass.