Identification and characterization of factor(s) that enhances the production of plant biomass-degrading enzymes by filamentous fungus and development of methods for its use

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Research objective

Among the natural resources, Japan is rich in forest resources, but produces almost no fossil fuels. Consequently, demand for utilization of natural energy, especially the effective use of plant biomass, has been increasing. However, hurdles remain in the practical use of plant biomass. One reason for this is the high cost of producing large quantities of enzymes required to break down plant cell walls. Plant cell walls primarily consist of three components: cellulose, hemicellulose, and lignin. Glucuronoyl esterase (GE) can cleave the bonds between xylan in hemicellulose and lignin. It is thought to loosen the overall structure of cell walls, thereby promoting their degradation.

Our group has long focused on this enzyme, demonstrating that NcGE, a GE derived from *Neurospora crassa*, can degrade a synthetic substrate that mimics the bond between the xylan side-chain glucuronic acid and lignin[1)]. Using this synthetic substrate, we identified key residues involved in substrate recognition based on structural predictions[2)]. In addition, we have worded to elucidate the mechanisms that induce the expression of plant cell wall-degrading enzymes in *N. crassa*. Recently, it was discovered that in a strain with high GE expression, the expression of cellulase and xylanase genes was enhanced, leading to increased enzyme activity in the culture medium. Furthermore, plant cell wall degradation products generated by GE *in vitro* contained substances that induced cellulase expression. This study aimed to analyze the substances and signaling pathways responsible for this increase in gene expression and to elucidate novel mechanisms regulating cellulase expression.

Methods

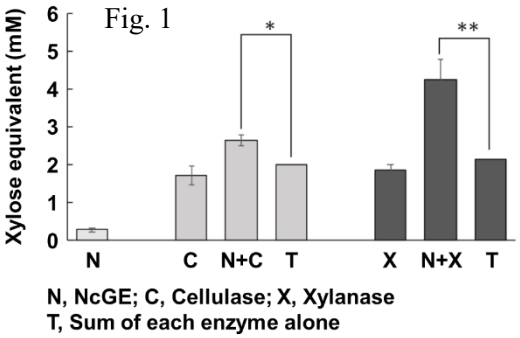
For heterologous expression of NcGE, the NcGE sequence was amplified from the *N. crassa* genome by PCR and cloned into the *Pichia pastoris* expression vector pPICzα. Transformants were cultured, and expression was induced by addition of methanol according to a standard protocol. NcGE was purified from the supernatant of the day 1 culture using a Ni^{2+} column. Purity was confirmed by Coomassie Brilliant Blue (CBB) staining and Western blotting. The substrate, mulberry wood powder, was prepared by grinding it in a ball mill and

then passing it through a 2 mm sieve, which was then reacted with NcGE. The supernatant was collected by centrifugation of the reaction mixture and NcGE was inactivated by heating before being added to the culture medium. After culturing *N. crassa*, the supernatant was sampled at various time points, and enzyme activity was measured. An *N. crassa* strain expressing NcGE was generated by ligating the NcGE sequence into vector pMF272, which possesses a constitutively strong *ccg-1* promoter, and transforming it into *N. crassa*. *N. crassa* gene knockout strains were obtained from the Fungal Genetic Stock Center.

Results

To investigate *in vitro* activity of NcGE, the heterologous production of NcGE was performed using *P. pastoris* as a host. The NcGE sequence was fused downstream of the budding yeast α-factor prepro sequence, and a strain expressing NcGE bearing a C-terminal myc and a His_6 tag was obtained. After induction with methanol, the supernatant was applied to a Ni^{2+} column, and the expression was confirmed by Western blotting using an anti-Myc antibody. NcGE thus obtained, commercial cellulase, and xylanase were reacted with mulberry wood powder, either individually or in combination, to evaluate their effects on the release of reducing sugars. The results showed that mixing NcGE with cellulase or xylanase released more reducing sugars than each enzyme alone, indicating that NcGE enhanced the effects actions of cellulase and xylanase[3)] (Fig. 1).

Fig. 1



Next, to explore the *in vivo* function of NcGE, a strain constitutively overexpressing NcGE under the control of the *ccg*-1 promoter was generated and cultured in a medium containing mulberry wood powder as the carbon source. The results showed that the activity of cellulase secreted into the medium was increased compared to that in the wild-type strain, and gene expression was also enhanced. This was speculated to be due to the action of NcGE, which likely induced the production of some inducer substances from plant cell walls, thereby enhancing the expression of cellulases.

To verify this hypothesis, mulberry wood powder was reacted with NcGE and added to the *N. crassa* culture medium, and enzyme activities in the supernatant were examined. The addition of NcGE-reacted mulberry wood powder significantly increased cellulase and xylanase activities compared to non-reacted wood powder (Fig. 2). This enhancing effect was attributed to the soluble fractions released from the mulberry wood powder, as it was lost upon dialysis, suggesting that it likely consisted of low-molecular-weight substances.

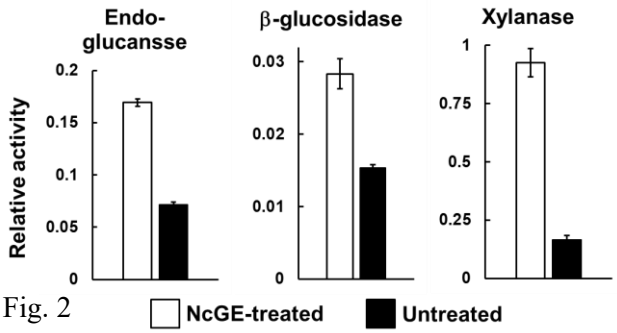


Fig. 2

To investigate the pathway by which NcGE induced cellulase expression, experiments were conducted using a gene-knockout strain. CLR-1 is a key transcription factor governing cellulase transcription in *N. crassa*, and cellulase expression is reduced in the knockout strain. As expected, when cultured in a medium containing mulberry wood powder as the carbon source, the Δ*clr-1* strain exhibited lower cellulase activity than that of the wild-type strain. However, when mulberry wood powder pre-reacted with NcGE was added to the medium, cellulase activity was nearly equivalent to that of the wild-type strain, particularly during the early stages of cultivation (Fig. 3). This suggests that at least part of the enhancement of cellulase expression by NcGE occurs via a novel pathway independent of CLR-1.

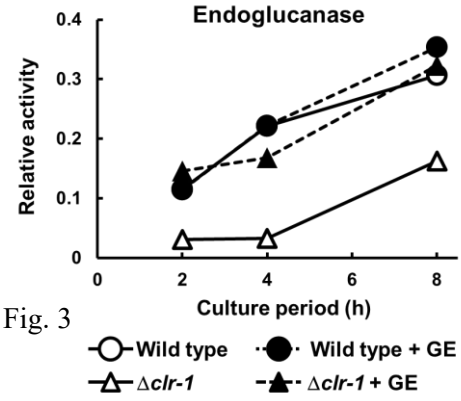


Fig. 3

Conclusion

In this study, we analyzed *in vitro* and *in vivo* functions of NcGE, a GE isolated from *N. crassa*. This revealed that NcGE promoted the degradation of plant cell walls by cellulases and xylanases; high expression of NcGE enhanced the expression of cellulases, and factors released during plant cell wall degradation by NcGE induced cellulase expression via a CLR-1-independent pathway. Future work will focus on identifying this cellulase induction factor and elucidating its intracellular signaling pathway. By regulating this pathway, we aim to enhance the cellulase production capacity and develop strategies to address the high costs associated with enzyme production.

References

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glucuronoyl esterase from the fungus *Neurospora crassa*: identification of novel consensus sequences containing the catalytic triad. *J. Gen. Appl. Microbiol.* **62**:217-224.

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