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Glucuronoyl esterase facilitates biomass degradation in *Neurospora crassa* by upregulating the expression of plant biomass-degrading enzymes

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Glucuronoyl esterase (GE) is a promising agent for the delignification of plant biomass since it has been shown to cleave the linkage between xylan and lignin *in vitro*. In this study, we demonstrate that *Nc*GE, a GE from *Neurospora crassa*, stimulates plant biomass degradation. *In vitro*, *Nc*GE synergistically increased the release of reducing sugars from plant biomass when added together with cellulase or xylanase. *In vivo*, overexpression of *Nc*GE in *N. crassa* resulted in an increase in xylanolytic activity. Consistently, elevated transcription of genes encoding the major plant biomass degrading-enzymes (PBDEs) was observed in the *Nc*GE-overexpression strain. Increased xylanolytic activity and transcription of PDBE genes were largely abolished when the transcription factors *clr-1*, *clr-2*, or *xlr-1* were deleted. Interestingly, the expression of some PBDE genes was increased when the hydrolysate of plant biomass by *Nc*GE was added to the culture medium. We propose that *Nc*GE boosts the production of PBDEs through the activation of key transcription factors, which is presumably caused by *Nc*GE-mediated generation of hypothetical inducer(s) from plant biomass.

Key Words: cellulase; glucuronoyl esterase; *Neurospora crassa*; plant biomass; xylanase

Abbreviations: GE, glucuronoyl esterase; *Nc*GE, GE from *Neurospora crassa*; PBDEs, plant biomass degrading-enzymes

Introduction

Albeit being the most abundant renewable resource in nature, plant biomass remains underutilized due to their recalcitrance and the high cost of conversion to valuable final products (Sánchez and Cardona, 2008). The enzymatic saccharification of physically- and chemically-pretreated lignocellulosic biomass mainly depends on the cooperative actions among cellulolytic and hemicellulolytic enzymes with different substrate specificities and modes of action (Bhattacharya et al., 2015). However, the covalent linkages between lignin and hemicellulose, such as phenyl glycosides, benzyl ethers, and esters contribute to the hierarchical complexity and recalcitrance of lignin-carbohydrate complexes (LCCs) that are hardly hydrolyzed by neither cellulases nor hemicellulases, hindering the complete decomposition of plant biomass (Du et al., 2013). In this regard, the unique function of glucuronoyl esterases (GEs) disconnecting the ester bond between 4-*O*-methyl-D-glucuronic acid residues of glucuronoxylans and aromatic alcohols of lignin may open a new way for biomass saccharification (Arnling Bååth et al., 2016;Monrad et al., 2018;Špániková and Biely, 2006).

GEs, first discovered and purified from the wood-rotting fungus *Schizophyllum commune* in 2006 (Špániková and Biely, 2006) belong to carbohydrate esterase family 15 (CE15) in CAZy database (http://www.cazy.org/) with nearly 500 entries up to date (Lombard et al., 2014). Besides some exceptions, they mainly originate from fungi and bacteria (Agger et al., 2017). Interestingly, not all fungi possess putative GE genes, indicating that GE is not essential for growth and the main carbon sources utilized by individual organisms are diverse. Also, GE-like sequences are not restricted to the strong wood-decaying fungi such as coprophilic fungi and white rot fungi. Although extensive studies have been performed as to their diversities,

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enzymatic properties, and structures (Mosbech et al., 2018;Pokkuluri et al., 2011;Špániková et al., 2007), physiological function of GEs in the process of degradation and utilization of plant biomass in microorganisms remains largely elusive.

The model filamentous fungus *Neurospora crassa* possesses a gene encoding GE (*NCU09445*, hereafter termed *NcGE*) in chromosome VII that is presumably involved in plant biomass degradation (Galagan et al., 2003;Huynh and Arioka, 2016). *N. crassa* is highly efficient in the degradation of plant biomass with its ability to synthesize and secrete a broad spectrum of carbohydrate-related hydrolytic enzymes that work on the substrates either individually or synergistically (Znameroski et al., 2012). Transcription and production these enzymes are regulated by the complex networks that involve the major transcription factors CLR-1 (cellulose degradation regulator 1, NCU07705), CLR-2 (cellulose degradation regulator 2, NCU08042), and XLR-1 (xylan degradation regulator 1, NCU06971) (Coradetti et al., 2012;Coradetti et al., 2013;Craig et al., 2015). The biomass hydrolysates generated by the actions of hydrolytic enzymes, such as cellobiose and xylose, could activate CLR-1 after they are incorporated into the cells (Sun et al., 2012). Afterward, the transcription of CLR-2 and XLR-1 are induced, which in turn leads to the triggering of lignocellulolytic responses in *N. crassa* (Hassan et al., 2019;Wu et al., 2020).

In this study, we first demonstrated that degradation of wood materials by cellulase and xylanase was synergistically increased by *Nc*GE *in vitro*. We then investigated the secreted xylanolytic enzyme activity and gene expression profiles of *Nc*GE-overexpression strain. Using the mutants deleted for the genes encoding key transcription factors, we showed that *clr-1*, *clr-2*, and *xlr-1* are required for *Nc*GE to promote the production of plant biomass degrading-enzymes (PBDEs). We finally demonstrated that *Nc*GE-treated biomass hydrolysate could stimulate the expression of PBDEs. These results suggest an *in vivo* role for *Nc*GE in generating hypothetical inducer(s) from plant biomass that boosts the expression of PBDEs.

Materials and methods

Strains and chemicals. *N. crassa* wild type (WT) strain (FGSC4200) and other *N. crassa* strains (Table S1) were cultivated in 1×Vogel's medium supplemented with 2.0% sucrose or wood extract. Deletion mutants were purchased from FGSC (Fungal Genetics Stock Center) or generated by crossing. *N. crassa* FGSC9717 (*mat A* Δ*mus-51*::*bar*+ *his-3*) strain was used for transformation. PrimeSTAR DNA polymerase and restriction endonucleases were purchased from TaKaRa. All other chemicals used were analytical grade reagents.

Production and purification of NcGE and Mut-NcGE. *Nc*GE was produced and purified as described (Huynh and Arioka, 2016). The catalytically inactive mutant of *Nc*GE (Mut-*Nc*GE) was generated as follows. The plasmid pPICZαA/NcGE (Huynh and Arioka, 2016) and the primers *Nc*GE-S211A-F and *Nc*GE-S211A-R (Table S2) containing the Ser211 to Ala mutation were used for inverse PCR. After amplification, the PCR product was digested by *Dpn* I, purified, and phosphorylated at the 5' end. Ligation was conducted by using the DNA ligation kit (Takara, Japan) to generate the mutant plasmid pPICZαA/Mut-NcGE. This plasmid was transformed to *P. pastoris* KM71H strain to obtain transformant used to produce Mut-*Nc*GE as inactive *Nc*GE.

GE activity assay. GE activity was examined by using the synthetic substrate benzyl methyl 4-*O*-methyl-α-D-glucopyranosiduronate (Huynh et al., 2018). The enzyme reaction was conducted in 100 mM sodium phosphate buffer (pH 5.5) at 30°C for 15 min. The hydrolysis product was analyzed by HPLC (Hitachi High-Tech Science, Tokyo, Japan) using Shiseido UG120A (4.6 mm×250 mm) at a temperature of 40°C. The mobile phase was acetonitrile:H_2O = 3:1 with the flow speed of 1 ml/min. The released benzyl alcohol was detected by UV detector at the wavelength of 254 nm.

Preparation of mulberry wood extract. To prepare the mulberry wood extract medium, the wood powder (20 g) was suspended in 400 ml distilled water, and then autoclaved at 121°C for 15 min. After removing the supernatant, the powder was resuspended in 500 ml distilled water. Afterward, the suspension was filtered with one-layer gauze and filled up to 1,000 ml by distilled water.

Synergistic effects of NcGE and hydrolytic enzymes. The substrate mulberry wood powder (concentrated from the wood extract) was suspended in 25 mM Tris-HCl (pH 7.0) to a concentration of 5 mg/ml. Then 50 μl (0.2 mg/ml) of purified *Nc*GE and 5 μl (10 mg/ml) of xylanase (from *Thermomyces lanuginosus*; Sigma X2753-10G) or 5 μl (10 mg/ml) of cellulase (from *Trichoderma reesei* ATCC 26921; Sigma 2730-50ML) were added either individually or together to the reactions to 20 μl wood extract followed by incubation at 30°C for 24 h. Reactions were stopped by heating at 100°C for 5 min, and the released reducing sugars were measured by the tetrazolium blue reagent method (Jue and Lipke, 1985).

Construction of $NcGE^{oex}$ and Mut-$NcGE^{oex}$ strains of N. crassa. Construction of $NcGE^{oex}$ and $Mut\text{-}NcGE^{oex}$ strains of *N. crassa* was performed as follows. The c-Myc and 6×His tags were fused at the C-terminus of the wild-type *NcGE* by fusion PCR (Table S2). The construct was then inserted into the *Sma* I site of the vector pMF272 to generate the overexpression plasmid pMF272-NcGE. The plasmid for overexpression of Mut-*Nc*GE, pMF272-Mut-NcGE, was constructed by using the same method as the construction of the plasmid for the expression of Mut-*Nc*GE. Transformation was conducted according to the method as described (Selker et al., 1997).

Secreted xylanolytic enzyme activity assay. Conidia of *N. crassa* (10^6) were inoculated into 20 ml 1×Vogel's medium containing mulberry wood, beechwood, or pinewood extract as the sole carbon source and grown at 30°C for 7 days with constant light. Culture supernatant (500 μl) was collected daily. To examine the relative xylanase activity, equal volumes of substrate beechwood xylan (0.5%, w/v) dissolved in 50 mM Tris-HCl buffer (pH 7.0) and culture supernatant were mixed and incubated at 30°C for

3 h. The reaction was stopped by heating at 100°C for 5 min, and the reducing sugars released were measured using the tetrazolium blue reagent following the method described previously (Inoue et al., 1997).

Transcriptional profile measured by qRT-PCR. For the expression profile analysis, seven-day-old conidia of *N. crassa* were collected and inoculated into 25 ml Vogel's sucrose medium at a final density of 10^5 conidia/ml. After 24 h incubation at 30°C with shaking, the mycelia were collected and washed with 100 ml of 1×Vogel's medium, and then transferred to and grown in 25 ml 1×Vogel's medium containing the mulberry wood extract as the sole carbon source under constant light for 18 h. When the effect of wood hydrolysates was examined, cells were cultured for 4 h. Total RNA isolation and cDNA synthesis were conducted following the standard procedures of ISOGEN (Nippongene, Japan) and PrimeScript cDNA synthesis kits (TaKaRa, Japan), respectively. Primers used for qRT-PCR analysis are listed in Table S2.

Preparation of NcGE-treated mulberry wood hydrolysate. Mulberry wood extract was condensed from 10 ml to 0.25 ml by centrifugation at 10,000 rpm for 5 min, to which 0.4 mg of purified *Nc*GE (2 mg/ml) and 0.3 ml of 25 mM Tris-HCl (pH 7.0) were added and the incubation was conducted at 30°C for 3 h followed by heating at 100°C for 5 min. After centrifugation at 3,000 rpm for 2 min, the supernatant containing hydrolysate was collected and filter-sterilized. Then the hydrolysate was added to the mulberry wood medium to grow *N. crassa* WT strain that were pregrown in 1×Vogel's medium supplemented with 2% sucrose for 24 h. After 4 h, total RNA was isolated followed by qRT-PCR analysis.

Results

NcGE stimulates biomass degradation by hydrolytic enzymes in vitro

First, we examined if recombinant *Nc*GE stimulates biomass degradation *in vitro*. As shown in Figs. S1A and S1B, C-terminally c-Myc and hexahistidine-tagged *Nc*GE produced in *P. pastoris* and purified by Ni-NTA chromatography displayed a 44 kD band in the Western blot. The activity assay using the synthetic substrate analog of glucuronic acid, benzyl methyl α-D-glucopyranosiduronate (Huynh et al., 2018), demonstrated that *Nc*GE produced the degradation product, benzyl alcohol (Fig. S1C). Since GE attacks the ester bond formed between 4-*O*-methyl glucuronoyl group of xylan and lignin alcohols in lignin-carbohydrate complexes (LCCs) (Mosbech et al., 2018;Špániková et al., 2007), we tested if *Nc*GE displays synergistic effects with commercial cellulase or xylanase in the deconstruction of mulberry wood extract. As shown in Fig. 1, only a small amount of reducing sugar was generated when *Nc*GE was acted alone to the substrate mulberry wood extract. However, we observed significant increase in the release of reducing sugars when *Nc*GE was acted together with cellulase or xylanase ('Theoretical' indicates the sum of *Nc*GE alone and cellulase or xylanase alone, respectively), indicating that *Nc*GE stimulates biomass degradation by cellulase or xylanase.

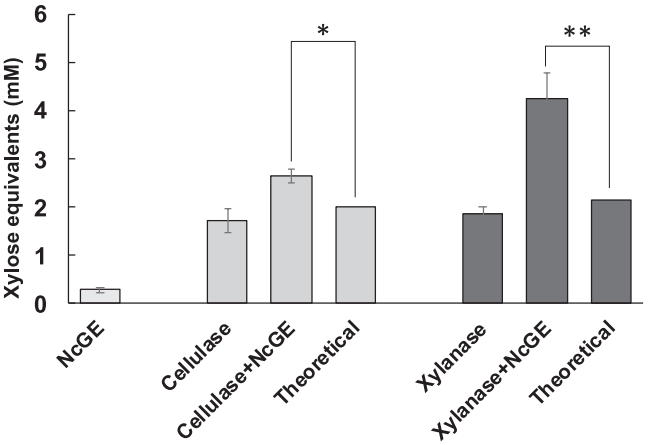

Fig. 1. Effect of *Nc*GE on biomass degradation *in vitro*

The amount of reducing sugars (xylose equivalents) released from mulberry wood extract treated with cellulase, cellulase plus *Nc*GE, xylanase, and xylanase plus *Nc*GE are shown. "Theoretical" indicates the sum of reducing sugars released by indicated commercial enzyme and recombinant *Nc*GE. Error bars, standard deviation (n≥3). *, $p \leq 0.05$ and **, $p \leq 0.01$ by two-tailed, unequal variance *t*-test. Cellulase, cellulase from *Trichoderma reesei* ATCC 26921; xylanase, xylanase from *Thermomyces lanuginosus*.

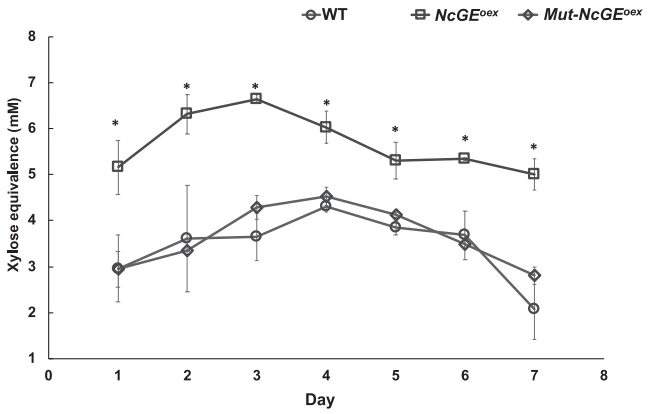

Fig. 2. Xylanolytic enzyme activities of wild type, *Nc*GE-overexpression, and Mut-*Nc*GE-overexpression strains grown in the medium containing wood extract as a sole carbon source

Xylanolytic enzyme activities in the culture supernatant of *N. crassa* wild-type (circles), $NcGE^{oex}$ (squares), and $Mut\text{-}NcGE^{oex}$ (diamonds) strains grown in mulberry wood extract medium. Samples were collected daily from the first to the 7th day. Error bars, standard deviation (n≥3). *, $p \leq 0.05$ by two-tailed, unequal variance *t*-test.

Overexpression of NcGE in N. crassa results in increased xylanolytic enzyme activity

Next, we examined whether *Nc*GE displays similar synergistic effect in the deconstruction of biomass *in vivo*. We constructed an *Nc*GE-overexpression strain ($NcGE^{oex}$) by transforming *N. crassa* with a plasmid carrying the *NcGE* sequence under the control of a constitutive promoter, P*ccg-1* (Figs. S2A and S2B). *Nc*GE was tagged with c-Myc and 6×His tags at the C-terminus. As shown in Fig. S2C, the band for *Nc*GE-Myc-His, the calculated size for which is 44.2 kDa, was detected in the Western blot of the culture supernatant obtained from $NcGE^{oex}$ strain grown in the medium containing mulberry wood extract as a sole carbon source (hereafter called mulberry wood medium). The

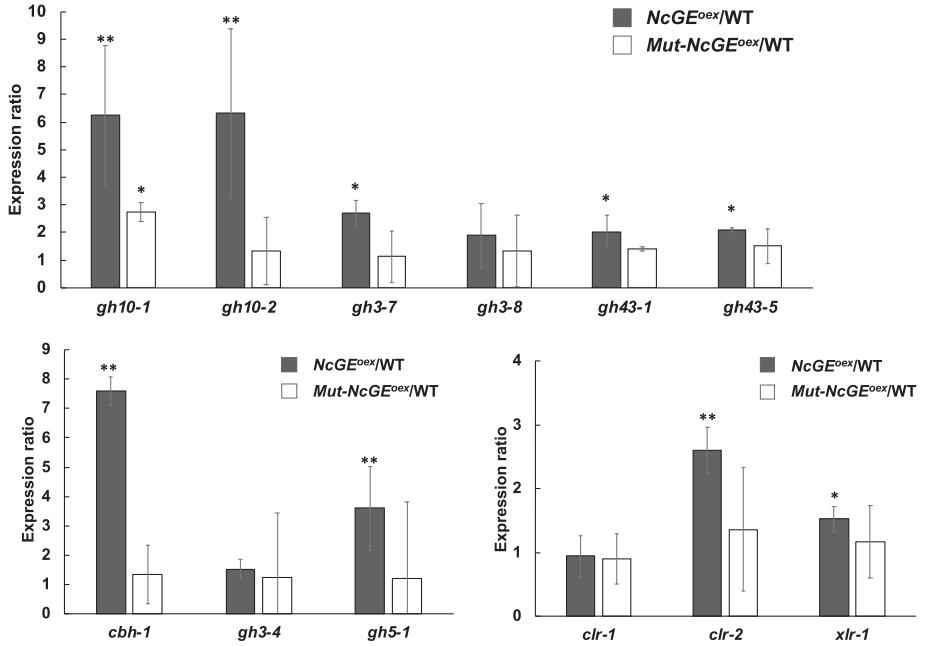

Fig. 3. Expression profiles of genes encoding PBDEs and transcription factors in the wild type, *Nc*GE-overexpression, and Mut-*Nc*GE-overexpression strains grown in the medium containing wood extract as a sole carbon source

N. crassa wild-type, *NcGE*^{oex}, and *Mut-NcGE*^{oex} strains were precultured in the sucrose-containing medium for 24 h, and then transferred to the medium containing mulberry wood extract as a sole carbon source. After 18 h the cells were collected and served for qRT-PCR analysis. Expression levels of genes coding for the major PBDEs and transcription factors in *NcGE*^{oex} (solid bars) and *Mut-NcGE*^{oex} (open bars) strains normalized by that in the wild type strain are shown. Error bars, standard deviation (n≥3). Statistical difference between *NcGE*^{oex} and wild type, and *Mut-NcGE*^{oex} and wild type is shown. *, $p \leq 0.05$ and **, $p \leq 0.01$ by two-tailed, unequal variance *t*-test.

activity assay of culture supernatant demonstrated an increase in the GE activity compared to the wild-type strain (WT; Fig. S2D), indicating that *Nc*GE was secreted to the culture medium. It should be noted that the band intensity in Fig. S2C does not correlate with the GE activity in Fig. S2D in the *NcGE*^{oex} strain at days 4 and 5. This could be due to the loss of C-terminal Myc tag from *Nc*GE-Myc-His protein.

We then evaluated the degradation of plant biomass by measuring the amount of reducing sugars generated in the culture supernatant of control and *NcGE*^{oex} strains grown in the mulberry wood medium for up to 7 days. However, we were unable to show significant difference in the amount of reducing sugars in the culture supernatant of two strains, presumably because the growth of *NcGE*^{oex} strain was poor compared to WT (Fig. S3). Growth retardation of *NcGE*^{oex} strain was also observed when grown on the medium containing sucrose as a sole carbon source. Although the precise reason for this growth inhibition is not clear, it is evident that it is due to the enzymatic activity of *Nc*GE, since the *Mut-NcGE*^{oex} strain overexpressing the catalytically-inactive mutant of *Nc*GE (see below) grew normally.

While we were monitoring the biomass degradation, we found that xylanase activity in the culture supernatant was higher in *NcGE*^{oex} strain compared to WT (Fig. 2). Similar results were obtained when the culture supernatants of cells grown in the beechwood and pinewood media were analyzed (Fig. S4). This was surprising, since, as stated above, the growth of *NcGE*^{oex} strain was poor compared to WT and no growth-based adjustment was conducted for the xylanase activity. To unequivocally show that the increase in xylanase activity is due to the enzymatic activity of *Nc*GE, we constructed a strain (*Mut-NcGE*^{oex}) overexpressing the catalytically inactive *Nc*GE mutant (Figs. S2A and S2B). This mutant was generated by mutating the key residue, S211 forming the catalytic triad Ser-His-Glu to alanine (Charavgi et al., 2013; Pokkuluri et al., 2011). To verify that mutant *Nc*GE (Mut-*Nc*GE) lacks the activity, the enzyme produced in *P. pastoris* and purified (Figs S1A and S1B right panel) was examined in the activity assay as described above, which showed that Mut-*Nc*GE almost completely lost the activity (Fig. S1C). *Mut-NcGE*^{oex} strain secreted the mutant *Nc*GE protein at a similar level to *NcGE*^{oex} strain (Fig. S2C), but the GE activity in the culture supernatant was nearly the same as WT (Fig. S2D). Finally, xylanase activity was not elevated in the *Mut-NcGE*^{oex} strain grown in the mulberry wood medium, indicating that GE activity is necessary for increase in the xylanase activity (Fig. 2). Similarly, no increase in the xylanase activity was observed in the *Mut-NcGE*^{oex} strain grown in the beechwood and pinewood media (Fig. S4).

NcGE induces the expression of PBDE genes in vivo

We then compared the expression levels of genes encoding xylanases and the other PBDEs (Fig. 3). Cells were precultured in the sucrose medium for 24 h, and then the

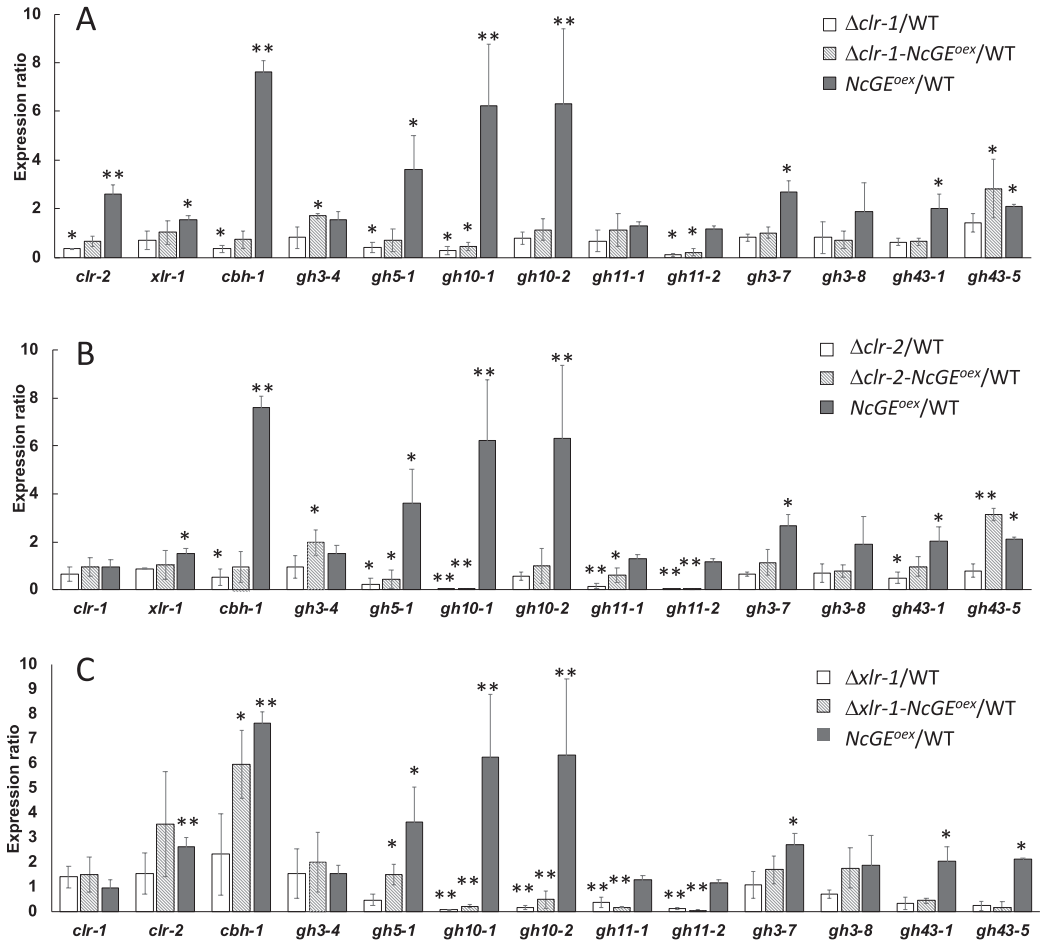

Fig. 4. Gene expression profiles in the strains deleted for transcription factors

Strains were precultured in the sucrose medium for 24 h, and then the mycelia were transferred to the mulberry wood extract medium and grown for 18 h with constant light followed by qRT-PCR analysis. Expression levels of the selected genes in (A) Δ*clr-1* and Δ*clr-1-NcGE*^{oex}; (B) Δ*clr-2* and Δ*clr-2-NcGE*^{oex}; (C) Δ*xlr-1* and Δ*xlr-1-NcGE*^{oex} normalized by that in WT strain are shown. Error bars, standard deviation (n≥3). Asterisks indicate that the values are significantly different from that in WT (*, $p \leq 0.05$ and **, $p \leq 0.01$ by two-tailed, unequal variance *t*-test).

mycelia were transferred to the mulberry wood medium and grown for 18 h with constant light. Results of quantitative reverse-transcription polymerase chain reaction (qRT-PCR) showed that two β-xylanase genes *gh10-1* (*NCU05924*) and *gh10-2* (*NCU08189*), as well as genes encoding GH3-7 (NCU09923; extracellular β-xylosidase), GH43-1 (NCU01900; intracellular β-xylosidase), and GH43-5 (NCU09652; intracellular β-xylosidase) exhibited significantly elevated transcription in the *NcGE*^{oex} strain (Li et al., 2015;Wang and Arioka, 2021). Furthermore, enhanced transcription of cellobiohydrolase gene *cbh-1* (*NCU07340*) and endoglucanase gene *gh5-1* (*NCU00762*) was observed in the *NcGE*^{oex} strain but not in the *Mut-NcGE*^{oex} strain. In contrast, the expression of genes encoding β-xylosidase GH3-8 (NCU00709) and β-glucosidase GH3-4 (NCU04952) as well as two GH11 β-xylanases GH11-1 (NCU02855) and GH11-2 (NCU07225) was not much affected (data not shown). Interestingly, the expression of *clr-2* and *xlr-1*, the key transcription factors responsible for the majority of cellulolytic and hemicellulolytic responses, was also upregulated in the *NcGE*^{oex} strain compared to the *Mut-NcGE*^{oex} strain, suggesting that overexpression of *Nc*GE boosted the transcription of PBDE genes via the increased expression of these transcription factors. The reason for elevated expression of *gh10-1* in the *Mut-NcGE*^{oex} strain compared to WT is not clear; overexpression of Mut-*Nc*GE itself could have generated a proteostatic stress that eventually led to the upregulation of *gh10-1*.

Increased expression of PBDE genes is dependent on CLR-1, CLR-2, and XLR-1

Given that CLR-1, CLR-2, and XLR-1 are critical transcription factors responsible for the vast majority of hydrolytic responses (Craig et al., 2015), we constructed the strains deleted for the genes encoding these transcription factors in the *NcGE*-overexpression background, and examined the transcription profile and secreted enzyme activity. As shown in Fig. 4A, increased expression of *clr-2* and *xlr-1* upon overexpression of *Nc*GE (solid bars) was abolished by disruption of *clr-1* (hatched bars). In accordance, the upregulation of *cbh-1*, *gh5-1*, *gh10-1*, *gh10-2*, *gh3-7*, and *gh43-1* in the *NcGE*^{oex} strain was not observed by the disruption of *clr-1*. Deletion of *clr-1* showed no effects on the increased expression of *gh43-5* in *NcGE*-overexpression background, suggesting that *Nc*GE

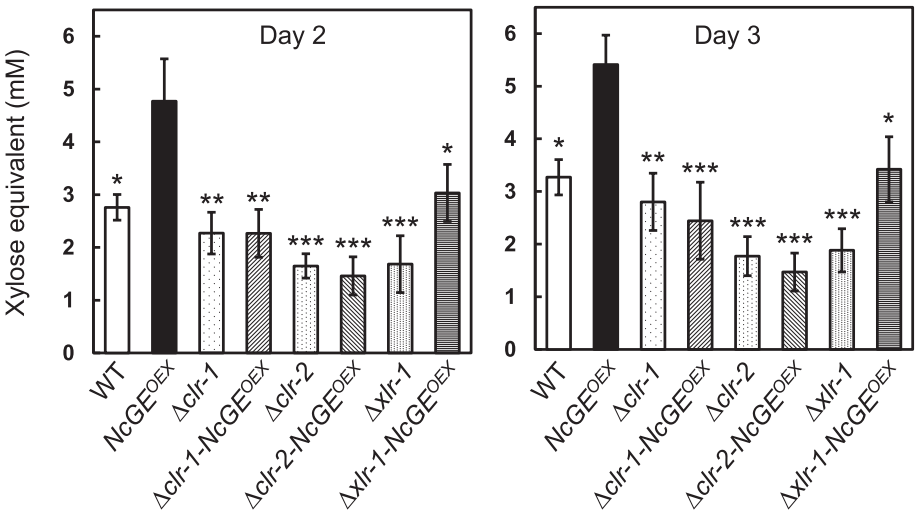

Fig. 5. Xylanolytic enzyme activities of strains deleted for transcription factors

Relative xylanolytic enzyme activities of various strains grown in the mulberry wood extract medium are shown. Error bars, standard deviation (n≥3). Statistical difference from *NcGE*^{oex} samples was examined by one-way analysis of variance followed by Tukey's post-hoc test. *, **, and *** represent p<0.05, 0.01, and 0.001, respectively (n=3).

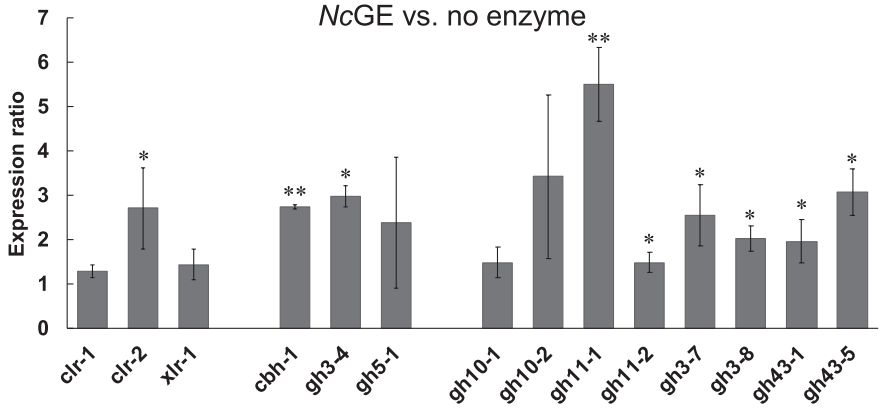

Fig. 6. Gene expression profiles in *N. crassa* grown in hydrolysate-containing medium

Expression profiles of genes encoding transcription factors and PBDEs in cells grown in mulberry wood medium containing *Nc*GE-treated wood hydrolysate. The data are normalized by those obtained from the cells grown in mulberry wood medium with no enzyme-treated hydrolysate. Error bars, standard deviation (n≥3). Asterisks indicate that the values are significantly larger than 1 (*, p≤0.05 and **, p≤0.01 by paired-samples t-test).

activates the expression of *gh43-5* independent of *clr-1*. An even severer expression defect was detected in the Δ*clr-2* mutant, in which the expression levels of the *β*-xylanase genes *gh10-1*, *gh11-1*, and *gh11-2* were almost zero (Fig. 4B, open bars). Increased expression of *cbh-1*, *gh5-1*, *gh10-1*, *gh10-2*, *gh3-7*, and *gh43-1* upon overexpression of *Nc*GE was also downregulated (Fig. 4B, hatched bars), indicating that *clr-2* is essential for *Nc*GE to stimulate the transcription of these genes. Similar to the strain overexpressing *Nc*GE in Δ*clr-1* background, increased expression of *gh43-5* in *NcGE*^{oex} strain was not affected by the deletion of *clr-2*, again suggesting that *Nc*GE-induced upregulation of these genes is not mediated by *clr-2*.

Deletion of *xlr-1* exhibited little impact on the transcription of *clr-1*, *clr-2*, and cellulolytic enzyme genes *cbh-1* and *gh3-4*, while the expression of *gh5-1* was slightly downregulated (Fig. 4C, open bars). Likewise, increased expression of *clr-2* and *cbh-1* upon overexpression of *Nc*GE was not much affected by the absence of *xlr-1*, whereas increased expression of *gh5-1* was slightly downregulated (Fig. 4C, hatched bars). In sharp contrast, four xylanase genes (*gh10-1*, *gh10-2*, *gh11-1*, and *gh11-2*) and two intracellular *β*-xylosidase genes (*gh43-1* and *gh43-5*) were significantly downregulated when *xlr-1* was deleted in both control and *Nc*GE-overexpression backgrounds. These results are consistent with the previous findings that the transcription factor XLR-1 is mainly responsible for the expression of hemicellulolytic, but not cellulolytic, genes (Sun et al., 2012), and indicate that increased expression of hemicellulolytic genes upon overexpression of *Nc*GE was dependent on *xlr-1*. Collectively, it was demonstrated that the upregulated expression of most of the cellulase/hemicellulase genes upon overexpression of *Nc*GE

was mediated by CLR-1/CLR-2 and/or XLR-1 systems.

We next investigated the secreted xylanolytic enzyme activity of *N. crassa* grown in the mulberry wood medium for 2 and 3 days (Fig. 5). Consistent with the gene expression profiles, results showed that the deletion of either of *clr-1* or *clr-2* in the *Nc*GE-overexpression background resulted in significant decrease in the xylanolytic activity. Interestingly, although the transcription of xylanase genes *gh10-1*, *gh10-2*, *gh11-1*, and *gh11-2* was dramatically decreased in both Δ*xlr-1* and Δ*xlr-1-NcGE*^{oex} strains to a similar extent (Fig. 4C), the decreased xylanolytic enzyme activity caused by the deletion of *xlr-1* was slightly restored by overexpression of *Nc*GE. It is of note, however, that we measured the amount of the reducing sugars in this assay. Since the substrate used to detect xylanase activity in this assay, beechwood xylan, is a relatively complex biomass material that may contain residual cellulose (Van Dyk and Pletschke, 2012), we speculate that enhanced expression of cellulase genes caused by overexpression of *Nc*GE might have contributed to generation of glucose or cellooligosaccharides and increase in the reducing sugars. For a similar reason, the apparent deficiency of 'xylanolytic enzyme activity' in Δ*clr-2* and Δ*clr-2-NcGE*^{oex} strains could be attributed to the downregulation of genes encoding both cellulolytic and xylanolytic enzymes. Collectively, these results further confirmed that *Nc*GE stimulates the transcription of hydrolytic enzymes through the key transcription factors *clr-1*, *clr-2*, and *xlr-1*.

NcGE-treated wood hydrolysate triggers the hydrolytic responses in N. crassa

We speculated that *Nc*GE secreted to the culture medium generated hypothetical inducer(s), which was incorporated to the cells and activated CLR-1/CLR-2/XLR-1, resulting in the expression of PBDEs. To test this hypothesis, the effect of hydrolysate prepared by treating mulberry wood extract with *Nc*GE in the gene expression was examined. As shown in Fig. S5, the recombinant *Nc*GE was added to condensed mulberry wood extract and incubated at 30°C for 3 h. The mock sample was prepared by incubating the condensed mulberry wood extract without *Nc*GE. Then the hydrolysates were collected, heat-treated, sterilized by filtration, and added to the mulberry wood medium that was used to cultivate the WT strain pre-grown for 24 in the sucrose medium. After 4 h, the cells were collected and the expression of major PBDEs was analyzed by qRT-PCR. Results demonstrated that the expression of *clr-2*, *cbh-1*, *gh3-4, gh11-1*, *gh3-7*, *gh3-8*, and *gh43-5* was upregulated by *Nc*GE-treated biomass hydrolysate (Fig. 6), suggesting that *Nc*GE-treated biomass contains substance(s) that triggers the expression of PBDEs in *N. crassa*. Although the genes upregulated by *in vivo* overexpression of *Nc*GE and by *Nc*GE-pretreated biomass are not fully consistent, at least for the genes upregulated in both experimental settings, e.g. *clr-2*, *cbh-1*, *gh3-7*, and *gh43-5*, it is likely that *Nc*GE produced in the *NcGE*^{oex} strain was directly responsible for the expression of these genes.

Discussion

Besides cellulases and hemicellulases, a family of newly-discovered enzymes, 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. 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 (Špániková and Biely, 2006), limiting our understanding of their physiological roles and potential application.

To gain insights into the physiological functions of GE 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, facilitated the deconstruction of plant biomass by mixed cellulases or xylanase. Then *N. crassa* wild type, *NcGE*^{oex}, and *Mut-NcGE*^{oex} strains were generated and used to evaluate the *in vivo* effects of *Nc*GE in the utilization of wood materials during growth. We found that overexpression of *Nc*GE in *N. crassa* resulted in increased production of xylanolytic enzymes as well as the expression of major PBDE genes including two *β*-xylanase genes *gh10-1* and *gh10-2*. Upregulated expression of PBDE genes as well as increased xylanolytic enzyme activity was abolished by the deletion of *clr-1*, *clr-2*, and *xlr-1*, suggesting that *Nc*GE secreted to the culture medium generated hypothetical inducer(s), which was incorporated to the cells and activated CLR-1/CLR-2/XLR-1. Indeed, application of *Nc*GE-treated biomass hydrolysate to *N. crassa* culture also triggered the expression of some PBDE genes, which was abolished by the deletion of *clr-1*, *clr-2*, and *xlr-1*, suggesting the generation of plant biomass-derived hypothetical inducer(s) by *Nc*GE.

It has been shown that oligosaccharides released from plant cell wall polymers and their derivatives trigger the expression of PDBE genes in fungi. For example, cellobiose induces the expression of endo-*β*-1,4-glucanase A gene in *Aspergillus nidulans* (Chikamatsu et al., 1999). In *N. crassa*, cellobiose, or a modified version of cellobiose, functions as an inducer of lignocellulolytic gene expression (Znameroski et al., 2012). In contrast, *Phanerochaete chrysosporium* uses cellotriose and cellotetraose, not cellobiose, as the inducers of cellobiohydrolases (Suzuki et al., 2010). In *Trichoderma reesei*, sophorose generated through the transglycosylation reaction of cellobiose catalyzed by an extracellular *β*-glucosidase potently induces the expression of cellulases (Sternberg and Mandels, 1979;Vaheri et al., 1979). Transglycosylation-dependent generation of inducer is also observed in the regulation of amylolytic gene expression in *Aspergillus* species; conversion of maltose derived from starch to isomaltose mediated by extracellular or intracellular *α*-glucosidases is essential for the activation of AmyR which is responsible for the induction of amylolytic enzymes such as *α*-amylase and glucoamylase (Kato et al., 2002a; Kato et al., 2002b; Suzuki et al., 2015; Ichikawa et al., 2021). Although it is unlikely that these glucose-related oligomers were generated from mulberry wood extract by *Nc*GE, it has been reported that *Cu*GE, a GE from the white-rot fungus

Cerrena unicolor, catalyzes the release of a mixture of acetylated aldouronic acids upon reaction on the lignin-enriched substrate prepared from raw birchwood (Mosbech et al., 2018). Similar products were also generated by other fungal GEs (Mosbech et al., 2019). If these are the substances that induce the expression of PDBE genes, then it would be intriguing to test if treating the mulberry wood extract by GH10 endo-xylanase together with *Nc*GE results in the enhanced expression of PDBE genes, since the release of aldouronic acids by *Cu*GE was synergistically promoted by the addition of GH10 endo-xylanase (Mosbech et al., 2018). Clarifying the molecular identity of hypothetical inducer(s) generated by *Nc*GE and the mechanism whereby they activate the expression of PDBE genes would be our next agenda.

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