Parallel proteomic and phosphoproteomic analyses reveal cellubiose-dependent regulation of lignocellulase secretion in the filamentous fungus *Neurospora crassa*

Bentao Xiong\(^1,2\) | Linfang Wei\(^1,3\) | Yifan Wang\(^1,3\) | Jinyu Li\(^1,3\) | Xin Liu\(^1,3\) | Yunheng Zhou\(^1,3\) | Panpan Du\(^1,3\) | Hao Fang\(^1,3\) | Johannes Liesche\(^1,3\) | Yahong Wei\(^1,3,4\) | Jisheng Li\(^1,3\) | Shaolin Chen\(^1,3,4\)

\(^1\)Biomass Energy Center for Arid and Semi-Arid Lands, Northwest A&F University, Yangling, Shaanxi, China

\(^2\)School of Life Sciences, Northwest University, Xi’an, China

\(^3\)College of Life Sciences, Northwest A&F University, Yangling, Shaanxi, China

\(^4\)Shaanxi Key Laboratory of Agricultural and Environmental Microbiology, Northwest A&F University, Yangling, Shaanxi, China

**Correspondence**
Shaolin Chen, Biomass Energy Center for Arid and Semi-Arid Lands, Northwest A&F University, Yangling, Shaanxi 712100, China.
Email: slc1916@nwsuaf.edu.cn

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**Abstract**
High cost of lignocellulases restricts the commercialization of biofuel and bio-product production from lignocellulosic biomass. Constitutively expressed lignocellulases are considered to degrade cellulose to release small amount of soluble cellodextrins such as cellubiose for further large-scale production of lignocellulases; however, the underlying mechanism remains to be elucidated. Here, a triple \(\beta\)-glucosidase mutant of the model fungus *Neurospora crassa*, which prevents rapid turnover of cellubiose and thus allows the disaccharide to induce lignocellulases, was applied to perform parallel analyses of proteome and phosphoproteome changes in response to cellubiose and Avicel cellulose. The results revealed shared proteome and phosphoproteome responses to cellubiose and Avicel, corroborating the idea that cellubiose mediates the regulation of lignocellulase expression and secretion. The results further suggest that this regulation is achieved at multiple levels, including epigenetic, transcriptional, post-transcriptional, translation, and post-translational. Proteome profiling revealed that the proteins upregulated by cellubiose and Avicel were over-represented in cellulose degradation and degradation product transport pathways. Phosphoproteome profiling revealed that the proteins differentially phosphorylated by cellubiose and Avicel were over-represented by the pathways such as transcriptional control, protein processing and export, cell wall biogenesis, and cellular signaling. Deletion mutation analysis further suggests that the ER chaperon protein Hsp70-6, the translocation complex subunit Sec66/Sec71, and the signal peptidase subunit Spc2 are involved in lignocellulase secretion, particularly translocation across the endoplasmic reticulum. Altogether, the results offer a new insight into how cellubiose mediates the regulation of lignocellulase expression and secretion, providing a potential strategy for the strain engineering to improve lignocellulase production.
INTRODUCTION

The plant cell wall is the most abundant biomaterial on the earth. Its major carbohydrate components are cellulose and hemicelluloses. Filamentous fungi are the main source of plant cell wall-degrading enzymes to produce biofuels and bio-products. Insoluble cellulose, for example, Avicel, can induce the production of cellulolytic enzymes. However, cellulose insolubility leads to shortcomings, such as difficult and complex fermentation operations and enzyme loss due to adsorption on cellulose (Gabelle et al., 2012). These drawbacks can be overcome by replacing insoluble cellulose with soluble inducers (Alessi et al., 2017; Rocha et al., 2016). Cellobiose, a soluble major cellulolytic product, can induce the production of lignocellulases (Wu et al., 2013; Znameroski et al., 2012). However, it is still not well understood how cellobiose induces the expression and secretion of cellulolytic enzymes. A systems understanding of the induction process will provide a foundation for rational optimization of strains for improved fermentation production of lignocellulases and other bio-based products (Liu & Qu, 2019).

Various pathways have been identified that modulate the production of cellulolytic enzymes, including transcription (Benocci et al., 2017), protein excretion (Kubicek et al., 2014; Starr et al., 2018), and cell morphology (Lin et al., 2018; Wang et al., 2017). Major cellulolytic transcription factors include CLR-1 and CLR-2 (Coradetti et al., 2012, 2013; Craig et al., 2015). CLR-3 is identified to be a repressor of CLR-1 activity in the absence of an inducer (Huberman et al., 2017). Another cellulolytic transcript factor CLR-4 is found to bind to the promoter region of CLR-1 (Liu et al., 2019). Glucose, derived from hydrolysis of cellobiose, triggers the repression of cellulolytic genes, a mechanism known as carbon catabolite repression (CCR). CCR is partially mediated by the transcription factor CreA/CRE-1 (Huberman et al., 2016), which is negatively regulated by the transcription factors COL26 and VIB1 (Xiong, Sun, et al., 2014). Recent studies suggest that the expression of cellulolytic genes is also subjected to regulation by the chromatin status, such as histone methylation (Li, Hu, Zhao, et al., 2019; Li, Hu, Zhu, et al., 2019).

Protein secretary pathway is another limiting factor of lignocellulase production (Fan et al., 2015; Starr et al., 2018). Recent studies suggest that lignocellulase excretion majorly occurs via the conventional ER-Golgi secretary pathway (Qin et al., 2017). The morphological state of filamentous fungi also has a large impact on the production of lignocellulases, particularly in submerged or solid-state fermentations (Cairns et al., 2019; Veiter et al., 2018). A major determinant of cellular morphology is the cell wall, which is regulated by the cell wall integrity (CWI) signaling pathway involving the cell surface sensors such as WSC-1 and MAP-kinase-mediated pathways (Verdín et al., 2019).

Post-translational modifications, particularly phosphorylation and dephosphorylation, may play a critical role in signal perception, transmission, and subsequent cellular responses during adaptation of filamentous fungi to plant cell wall polysaccharides (Horta et al., 2019; Rodriguez-Iglesias & Schmoll, 2019; Schmoll, 2018; Xiong, Coradetti, et al., 2014). Phosphorylation of CRE-1 has been found to be required for binding to its target sequences in Trichoderma reesei and for triggering CCR (Cziferszky et al., 2002; Han et al., 2020). Six non-essential protein phosphatases were found to be positive or negative regulators for lignocellulase production in T. reesei (Rodriguez-Iglesias & Schmoll, 2019). Protein kinases were also found to participate in regulating lignocellulase production (Lin et al., 2019; Wang et al., 2017).

Currently, an acceptable model for the induction of lignocellulases is that extracellular cellulose is degraded by constitutive cellulases to release small amount of soluble celdodextrins such as cellobiose, which act as inducers for further large-scale production of lignocellulases (Kubicek et al., 2009). Understanding how cellobiose regulates lignocellulase production, we applied a triple β-glucosidase mutant of the model fungus Neurospora crassa that prevents rapid turnover of cellobiose and thus allows the disaccharide to induce lignocellulase production to profile and compare the proteomes and phosphoproteomes treated with cellobiose and Avicel cellulose. The derived regulatory pathways should have an impact on a systems engineering approach for strain improvement to produce lignocellulases (Li et al., 2017).

MATERIALS AND METHODS

2.1 Cell culture

N. crassa WT strain OR74A (FGSC2489) and available single-gene deletion mutants were obtained from the Fungal Genetics Stock Center (http://www.fgsc.net/). The Δ3βG mutant strain (Znameroski et al., 2012) of N. crassa was inoculated onto slants of Vogel’s minimal medium (VMM; Vogel, 1956) with 1.5% agar and 2% sucrose and grown for...
3 days at 30°C in the dark followed by 7 days at 25°C in light. Conidia were inoculated into 100 ml of liquid VMM with 2% (w/v) sucrose at 10⁶ conidia/ml and grown at 25°C in constant light and shaking for 16 h. Mycelia were harvested by filtrating and washed with VMM without a carbon source, followed by growth in fresh VMM with either 0.5% sucrose, 0.2% cellobiose, 0.5% Avicel® PH-101 (Sigma Aldrich), or with no carbon source added, as described previously (Liu et al., 2020).

### 2.2 Measurements of secreted proteins and cellulase activity

Total secreted proteins in the culture filtrates of wild type and mutant *N. crassa* strains were determined using the Bio-Rad DC Protein kit (Bio-Rad). Bovine serum albumin (BSA) was used as the standard. CMCase activity in culture supernatants of *N. crassa* strains was measured in microplates (96-well PCR plate) as described (Castro et al., 2014; Xiao et al., 2005). Briefly, 30 μl of 1% carboxymethylcellulose (CMC) in sodium acetate buffer (pH 4.8) was added to 30 μl of sample. Reaction was performed at 50°C for 30 min. After the addition of 60 μl of DNS, an additional heating step was carried out at 95°C for 5 min. At least three biological replicates were performed for each individual experiment. One enzyme unit is equivalent to the conversion of 1 μmol of reducing sugar per minute.

### 2.3 Protein digestion and TMT labeling

Mycelia were harvested at 4 h after media shift by filtering through Miracloth and immediately frozen in liquid nitrogen. Frozen mycelia were ground into fine powder with mortar and pestle. The powder (~100 mg) was suspended in ice-cold lysis buffer (250 mM sucrose, 20 mM HEPES, 0.1 mM EGTA, 1 mM EDTA, pH 7.4 adjusted with 1 N KOH, 4°C) with freshly added 1X cOmplete™ Protease Inhibitor Cocktail and 1X PhosSTOP Phosphatase Inhibitor Cocktail. Cell lysate was centrifuged for 20 min at 20,000 g at 4°C to remove cell debris. Proteins were reduced by adding dithiothreitol (DTT) to a final concentration of 10 mM and incubating for 30 min at 60°C and alkylated with iodoacetamide (final concentration 20 mM) for 30 min in the dark at room temperature. Excess iodoacetamide was quenched by the addition of DTT to a final concentration of 20 mM. Five volumes of acetone (precooled at −20°C) were added to the protein solution and were incubated at −20°C for 3 h, before being centrifuged at 8000 g for 10 min. Protein pellet was washed with precooled 90% acetone and air-dried.

An aliquot of 25–100 μg of acetone-precipitated protein was suspended in 100 mM triethylammonium bicarbonate (TEAB), crushed into fine particles, and incubated at 37°C overnight after trypsin was added at a protein/trypsin ratio of 40:1 (w/w). Digested peptides were labeled using TMT 6-plex isobaric mass tagging reagents (Thermo Scientific) according to the manufacturer instructions. The peptide solution was centrifuged for 10 min at 20,000 g to remove any insoluble material. Peptides were concentrated and desalted on a peptide trap column (Michrom) and eluted from the trap with 1–2 trap volumes of buffer B (80/20/0.1 acetonitrile/H2O/TFA). Peptide solution was acidified with 10% trifluoroacetic acid (TFA), centrifuged, and dried to about 5 μl by vacuum centrifugation.

### 2.4 Phosphopeptide enrichment

Phosphopeptide enrichment was performed using the same procedure as described in a previous study (Ficarro et al., 2009). Briefly, magnetic Ni-NTA agarose beads were firstly washed with water and treated with 100 mM EDTA (pH 8.0). Beads were then washed with water, treated with 10 mM FeCl3, and washed again with water and methanol:acetonitrile:0.01% acetic acid (1:1:1). Subsequently, tryptic peptides in buffer B were added to the beads and phosphopeptide capture proceeded for 30 min. After washing with buffer B, the beads were finally eluted with 1:20 ammonia/water for 30 min.

### 2.5 LC-MS and data analysis

The liquid chromatography-mass spectrometry (LC-MS) and data analysis were performed as described in our previous study (Liu et al., 2020). An appropriate volume of 5% acetonitrile (v/v) solution with 0.1% (v/v) trifluoroacetic acid was added to the dried peptide samples. The samples were fractionated on an EASY-Spray column (50 cm × 75 μm ID, PepMap RSLC C18, 2 μm) using an EASY-nLC 1200 system (Thermo Scientific) coupled to an Orbitrap Fusion Tribrid mass spectrometer. Mobile phases A and B were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid, respectively. A 210 min gradient was used with a flow rate of 200 nl min⁻¹.

For MS analysis, a data-dependent method of synchronous precursor selection (SPS)-MS3 was used (Table S1). Relative quantification of protein abundance between two different media, that is, no carbon versus sucrose (NC vs. Suc), cellobiose versus no carbon (CB vs. NC), and Avicel versus no carbon (AV vs. NC) conditions, was performed for each replicate.

Proteome Discoverer 1.4 (Thermo Scientific) was used to search MS/MS spectra against the *N. crassa* strain OR74A database from NCBI and the resulting peptide hits were filtered for maximum 1% FDR. The TMT sixplex quantification
method was used to calculate the reporter ratios with mass tolerance ± 10 ppm. Only peptide spectra containing all reporter ions were designated as “quantifiable spectra.” Spectra of the non-TMT, standard peptides were analyzed manually. For datasets from the MS3 method which contained both MS2 and MS3 spectra, CID (MS2) spectra were used for identification and HCD (MS3) spectra were used for quantification.

The reporter ion intensities in the spectrum of each TMT-labeled peptide represent the total quantification signal for that peptide. The statistical significance of results was evaluated with the Student’s t test and a p-value of less than 0.05 was considered statistically significant. Gene ontology enrichment was obtained from DAVID Bioinformatics Resources 6.8 (Huang et al., 2008) and the FungiFun2 tool (Priebe et al., 2014). Venn diagram was constructed using the web-based tool (http://bioinformatics.psb.ugent.be/webtools/Venn).

The data that support the findings of this study are available in the supplementary material of this article.

3 | RESULTS AND DISCUSSION

3.1 | A shared proteome and phosphoproteome response to cellobiose and Avicel cellulose

To examine and compare the early adaptation of N. crassa to Avicel cellulose and cellobiose, the same growth conditions as previously used for transcriptome analysis (Xiong, Coradetti, et al., 2014; Znameroski et al., 2012, 2014) were applied for a parallel quantitative proteome and phosphoproteome analysis. The parallel analysis was performed 4 h after the Δ3βG mutant was switched to Avicel (AV) or cellobiose (CB)-containing medium, while the samples from the cultures grown on sucrose (Suc) or no carbon (NC) were used as comparative controls (Figure 1a). The efficiency of transcription in the Δ3βG mutant in response to cellobiose is similar to that of the wild type to Avicel (Xiong, Coradetti, et al., 2014; Znameroski et al., 2012, 2014), and the identity and amount of proteins secreted by the Δ3βG mutant on cellobiose mimic that by the wild type on Avicel (Znameroski et al., 2012).

Both MS2 and MS3 acquisition were used for quantitative profiling of N. crassa proteomes and phosphoproteomes (Figure 1). As MS3 eliminates the ratio distortion in isobaric multiplexed quantitation and improves the sensitivity, resolution, and dynamic range of MS analysis (Ting et al., 2011), only the results derived from MS3 were analyzed and presented. The protein abundances derived from quantitative proteome profiling were used to calibrate phosphopeptide ratios (Figure 1a), which allow differential phosphorylation to be distinguished from altered protein abundances (Wu et al., 2011). We set the criteria for downregulation to a TMT ratio ≤0.77 (fold change −1.3) and upregulation was determined with a TMT ratio of ≥1.3 (fold change 1.3) with p-values less than 0.05. This analysis identified 12,855 phosphopeptides, among which 1955, 1474, and 1832 showed differential abundances with a 1.3-fold change cutoff in response to cellobiose (CB vs. NC), Avicel (AV vs. NC), and carbon starvation (NC vs. Suc; Table S2), respectively, corresponding to 1031, 830, and 1011 differentially phosphorylated proteins (Figure 1b). Among the differentially phosphorylated proteins in response to cellobiose and/or Avicel, 42 were the members of the Ire1/HAC1 governed unfolded protein response (UPR) regulon (Fan et al., 2015).
A shared phosphorylation response to cellobiose and Avicel was identified as reflected by that 728 phosphoproteins exhibited differential phosphorylation in response to both cellobiose and Avicel (Figure 1b). A shared proteome response to cellobiose and Avicel was also shown by our quantitative proteome analysis (Liu et al., 2020). Gene Ontology (GO) enrichment analysis revealed that the differential phosphoproteome in response to cellobiose and Avicel was over-represented by the pathways related to cellular signaling, transcriptional control, and protein translocation and excretion (Figure 2; Table S3), while the proteome with increased abundance was over-represented by cellulose catabolism, carbohydrate metabolism, and sugar transport (Liu et al., 2020).

### 3.2 Cellobiose-dependent transcriptional and epigenetic regulation of cellulolytic responses

Over 300 transcription factors (TFs) have been annotated in the genome of *N. crassa* (Borkovich et al., 2004; Carrillo et al., 2017; Weirauch et al., 2014). Fifty-nine of the 300 transcription factors were differentially phosphorylated in response to cellobiose and/or Avicel cellulose (Table S4). Increased phosphorylation was observed on the S108 residue of the essential cellulase transcription factor Clr1 in response to cellobiose and Avicel (Table 1). Increased phosphorylation was also identified for another cellulase transcription regulator Clr3 on S760, S767, and S789 (Table 1). Another transcription factor Vib1, which is essential for cellulolytic response in *N. crassa* and acts as a link between glucose signaling and CCR (Xiong, Sun, et al., 2014), showed increased phosphorylation on S96/Y97, S532, and S535 in response to cellobiose and Avicel (Table 1). The transcription factor Res2, which is involved in the UPR or endoplasmic reticulum (ER) stress response under cellulolytic conditions (Fan et al., 2015), showed increased phosphorylation on S291 (Table 1). By contrast, decreased phosphorylation was found for Cre1, a key carbon catabolite repression (CCR) regulator. The differential phosphorylation on the cellulolytic transcription factors may reflect differential regulation of lignocellulase expression and secretion: dephosphorylation of Cre1, along with phosphorylation of Vib1, may activate lignocellulolytic gene expression indirectly by relieving CCR (Xiong, Sun, et al., 2014), while phosphorylation of Clr1, Clr3, and Res2 may have direct effects on activating lignocellulase expression and secretion (Fan et al., 2015; Samal et al., 2017).

Multiple phosphorylation sites of Cre1/CreA have been identified in *N. crassa* (Figure S1) and other filamentous fungi (de Assis et al., 2021; Horta et al., 2019) and characterized. However, only dephosphorylation on S6 and S211 was identified in response to cellobiose and Avicel (Table 1), and none of them were characterized in previous studies. Quantitative profiling of *T. reesei* phosphoproteome under cellulolytic conditions in a previous study did not show differential phosphorylation on CreA (Nguyen et al., 2016), probably due to lower precision and more missing values of label-free quantitation than MS3-based TMT approach (O’Connell et al., 2018).

In addition to transcription, some epigenetic factors, such as Set1, Set2, Set4, and Set8, were also differentially phosphorylated in response to cellobiose and Avicel (Table 1). Recent studies suggest that disruption of *set2* in *Penicillium oxalicum* resulted in an extensive upregulation of lignocellulase production, while disruption of *set1* led to downregulation (Li, Hu, Zhu, et al., 2019). Further studies will be required to determine how the identified phosphorylations modulate the functions of the epigenetic and transcription factors during cellulolytic responses.

### 3.3 Cellobiose-dependent post-transcriptional, translational, and post-translational responses during early adaptation to cellulose

Correlation analysis suggests that there is only a moderate correlation between changes in protein and mRNA levels

**FIGURE 2** Comparison of the functional categories enriched in differential proteome and phosphoproteome in response to cellobiose (CB vs. NC) and Avicel (AV vs. NC)
| NCU no. | Annotation or domain | Phosphopeptide | PhosphoRS Site probability | CB versus NC | AV versus NC |
|---------|---------------------|----------------|---------------------------|--------------|--------------|
|         |                     |                |                           | Log2 fold change | p-value | Log2 fold change | p-value |
| **Cellular signaling** |                     |                |                           |               |            |               |          |
| NCU05853 | Cellubionic acid transporter (Sut12/Cbt1/Clp1) | rS[pT]NENk | T525: 100.0 | 0.95 | 0.000 | 0.80 | 0.000 |
| NCU03253 | G protein-coupled receptor (Gpr8) | nNAPGEyGGT[pS]PGPR | S477: 89.4 | -0.60 | 0.000 | -0.26 | 0.016 |
| NCU08377 | Adenylate cyclase (Cr1) | qT[pT]APGFTTk | T625: 100.0 | -1.24 | 0.000 | -1.36 | 0.000 |
| NCU06240 | cAMP-dependent protein kinase type 3 (Pkac1) | (o)W(t)LCGTPDYALEPVSnk | T377: 47.8; T379: 47.8 | -1.21 | 0.000 | -1.27 | 0.000 |
| NCU03860 | cAMP-regulated phosphoprotein family protein (Igo1) | kYFD[pS]GDYAmSk | S47: 100.0 | -1.38 | 0.000 | -1.35 | 0.000 |
| NCU03269 | Serine/threonine-protein phosphatase 2A activator 2 (Rrd2) | sGIGTGQG[pS]PAASGQPISk | S18: 100.0 | -1.09 | 0.000 | -0.47 | 0.001 |
| NCU03200 | Serine/threonine protein kinase-10 (Stk10) | sGVA[pT]PQPDLHdk | T81: 100.0 | -0.94 | 0.000 | -0.31 | 0.007 |
|         |                     | [pS]GVA[pT]PQPDLHdk | S77: 100.0; T81: 100.0 | -1.50 | 0.000 | -0.70 | 0.000 |
|         |                     | lPGlmSYFNQSGAS[pT]PTRY | T105: 97.1 | -0.77 | 0.000 | -0.33 | 0.001 |
|         |                     | qS[pS]NTSL[pT]DFNTR | S237: 99.4; T242: 93.5 | -1.04 | 0.000 | -0.51 | 0.000 |
|         |                     | sDTDVSYFDPEFTNLANNG[pS] LNER | S652: 100.0 | 0.61 | 0.000 | 1.38 | 0.000 |
| NCU07378 | Serine/threonine protein kinase-12 (Stk12) | sLILPGTIpS]PR | S606: 100.0 | 0.61 | 0.000 | 0.64 | 0.000 |
|         |                     | sT[pS]LDVHAR | S894: 100.0 | -0.91 | 0.000 | -0.73 | 0.000 |
|         |                     | lED[pS]PVSASGSSk | S1657: 100.0 | 1.06 | 0.000 | 0.63 | 0.001 |
| NCU02815 | Osmotic-1 (Os1) | gLTAADSLVSGLE[pS]PSIVTADk | S1270: 100.0 | -1.94 | 0.000 | -1.41 | 0.000 |
| NCU06544 | Protein kinase C (Pkc1) | q[pS]VDDFEGYGAP[pS]PPpk | S87: 100.0; S98: 100.0 | -1.79 | 0.000 | -0.95 | 0.000 |
| **Transcriptional and epigenetic control** |                     |                |                           |               |            |               |          |
| NCU07705 | Cellulose degradation regulator-1 (Cir1) | aP[pS]EESTNPLk | S108: 100.0 | 0.88 | 0.000 | 1.08 | 0.000 |
| NCU05846 | Cellulose degradation regulator-3 (Cir3) | rS[pS]AIk | S760: 100.0 | 3.16 | 0.000 | 2.43 | 0.000 |
|         |                     | [pS]VDGGLkDEEQEkl | S767: 100.0 | 3.17 | 0.000 | 2.64 | 0.000 |
|         |                     | ek[pS]PLASlk | S789: 100.0 | 1.43 | 0.000 | 0.94 | 0.000 |
| NCU08807 | Transcription factor (Cre1) | vQ[pS]AVDFSNNLNPSESTAEk | S6: 100.0 | -1.44 | 0.000 | -1.44 | 0.000 |
|         |                     | sSAGSQ[pS]GPDISLlAR | S211: 100.0 | -0.54 | 0.000 | -0.43 | 0.000 |
| NCU03725 | Transcription factor (Vib1) | (SY)SQVDQTPYTEMVQDLR | S96: 48.8; Y97: 48.8 | 1.14 | 0.000 | 0.84 | 0.000 |
|         |                     | hG[pS]HG[pS]LTNETASGyKR | S532: 100.0; S535: 99.9 | 1.00 | 0.000 | 0.85 | 0.001 |
| NCU02724 | HLH transcription factor (Res2) | rE[pS]HNLYVER | S291: 100.0 | 0.63 | 0.000 | 0.44 | 0.007 |
| NCU01206 | Histone-lysine N- methyltransferase, H3 lysine-4 specific (Set1) | aAGIEDIEG[pT]PDAEAk | T940: 100.0 | 0.82 | 0.000 | 0.65 | 0.000 |

(Continues)
| NCU no.  | Annotation or domain                              | Phosphopeptide                                      | PhosphoRS Site probability | Log2 fold change | p-value | CB versus NC | Log2 fold change | p-value | AV versus NC | Log2 fold change | p-value |
|---------|---------------------------------------------------|-----------------------------------------------------|----------------------------|-------------------|---------|--------------|-------------------|---------|--------------|-------------------|---------|
| NCU00269 | Histone-lysine N-methyltransferase, H3 lysine-36 specific (Set2) | eDSTTEDSIP[pS] PPPPPPPPTDTPLTEEER | S850: 94.3 | 1.55 | 0.000 | 1.79 | 0.000 |
| NCU04389 | Histone methytransferase-4 (Set4)                 | tQSSSTIGPATD[pS]R | S512: 100.0 | 0.75 | 0.000 | 0.82 | 0.000 |
| NCU01973 | Histone methytransferase-8 (Set8)                 | rL[pS]NDYVPSDSAPATGQHR | S691: 100.0 | 1.05 | 0.000 | 0.64 | 0.002 |

**Translational control**

| NCU01468 | Translation initiation factor eIF2B subunit delta | rP[pS]VAIk | S118: 100.0 | 0.61 | 0.002 | 0.56 | 0.003 |
| NCU00457 | Translation initiation factor 4B (eIF4B)          | tV[pS]EAPAEAGSTASTDSk A[pS]PAAWPGEGR qD[pS]RP RR | S333: 100.0 | −1.72 | 0.000 | −1.29 | 0.000 |
| NCU03717 | Translation initiation factor 4E (eIF4E)          | gL[s]GLSQ[s]NDA[pS]VSSPAAPER | S13: 49.8; S18: 49.8; S22: 92.1 | −1.43 | 0.000 | −0.58 | 0.000 |
| NCU09546 | Translation initiation factor eIF4E3              | tNPFNSITPPGLV[pS] PTAGASSAFGLGSAFASFGSAk | S62: 85.9 | 0.94 | 1.67 | 0.000 |
| NCU00366 | Eukaryotic translation initiation factor 5 (eIF5)  | qNGGdkEG[pS]GDEG[pS]VSSPAIEDk | S172: 100.0; S178: 100.0 | 0.69 | 0.000 | 0.36 | 0.030 |
| NCU03061 | Translation initiation factor RLI1                | ISGNYFILDDPEk(ss) | S606: 50.0; S607: 50.0 | −1.06 | 0.000 | −0.37 | 0.000 |

**Protein translocation and excretion**

| NCU03819 | COPII coat assembly protein (Sec16)               | aD[pT]DEPSDVASK r[pT]PPQSLTVQPVTSPQR sQ[pT]Q[pS]PGALYGNR | T166: 97.7 | −0.72 | 0.000 | −0.57 | 0.000 |
| NCU06738 | Protein transporter (Sec31)                        | nTPSVAPLT[pS] PFPQPGIASSPPAGPFQ QGAP[pT]TPPPPk tA[pS]PYNPSPASAAAPPANR nVGAPppGPpRRSSSV(ss)Na(t)Np(y) APRPPAAGLQPTYGVPPAPR | S958: 97.7; T981: 100.0 | −0.39 | 0.005 | −0.43 | 0.000 |
| NCU08379 | Protein transport protein subunit beta (Sec61)     | aSSPSSPPAGGSTGVSSARP(ss) PPPPGGAR | S26: 50.0; S27: 50.0 | −0.40 | 0.005 | −0.33 | 0.006 |

(Continues)
in *N. crassa* in response to cellulose (Figure 3; Liu et al., 2020). Comparison of proteome and transcriptome changes revealed that the proteins with increase in both protein abundance and mRNA levels of their encoding genes were primarily associated with cellulose degradation and degradation product transport (Figure 2; Table S5; Liu et al., 2020), corroborating the finding that cellulose degradation and utilization are controlled primarily at transcriptional level (Huberman et al., 2016). It was also noted that some proteins showed increased abundance in early response to cellobiose and Avicel but no change of the mRNA levels of their encoding genes (Figure 3; Table S5; Liu et al., 2020). These proteins were associated with post-transcriptional (e.g., mRNA processing, rRNA processing, and ribosome biogenesis) and post-translational processes (e.g., protein processing and export; Table 2).

In addition, the proteins associated with protein translation initiation also showed differential phosphorylation in response to cellulose and Avicel, including elf2B, elf4B, elf4E, elf4E3, elf5, and RL1 (Table 1). Previously identified phosphorylation of elfs in eukaryotic cells is primarily limited to the response to intracellular stresses (Proud, 2019). In yeast, glucose starvation induces phosphorylation of elf2α on S51, which reduces the level of active elf2, resulting in inhibition of translation initiation (Janapala et al., 2019), a key control step in protein synthesis. Here, we found that cellobiose and Avicel treatments upregulated the phosphorylation of elf2B, elf4E3, and elf5, but downregulated the phosphorylation of elf4B, elf4E, and RL1 (Table 1). It is possible that these phosphorylation events are particularly involved in activating translation initiation of lignocellulases in early response to cellulose.

Altogether, the results suggest that cellobiose regulates the early adaptation of *N. crassa* to cellulose at post-transcriptional, translational, and post-translational levels (Collier et al., 2020; Cziferszky et al., 2002; Li, Pang, et al., 2019; Lin et al., 2019; Liu et al., 2020). Regulatory roles of these newly identified phosphorylations in these processes need to be examined in further studies.

### 3.4 Cellobiose-dependent protein processing and export for cellulolytic responses

In filamentous fungi, high protein secretion capacity requires an extremely efficient system for protein folding and transport. Compared with transcription factors, far less is known of how lignocellulases are secreted into the extracellular environment (Huberman et al., 2016). In general, when the folding capacity of the ER is exceeded, the UPR pathway is triggered, allowing cells to mitigate and cope with the ER stress. In yeast, the only identified ER stress sensor is Ire1, an ER-resident transmembrane protein kinase receptor. In ER-stressed yeast cells, the HAC1 mRNA is spliced by activated Ire1 and then translated into a transcription factor, which functions in UPR transcriptional induction (Walter & Ron, 2011). In *N. crassa*, HAC1 has been shown to be involved in the UPR response and is necessary for growth on cellulose (Montenegro-Montero et al., 2015). The targets of the Ire1-HAC1-governed UPR regulon have been identified in *N. crassa* stressed by lignocellulase production (Fan et al., 2015). Forty-two of the 251 Ire1-HAC1 regulon members were differentially phosphorylated in response to cellobiose and/or Avicel (Figure 1c; Table S6). The differentially phosphorylated members include Sec16, Sec31, Sec63, and Sec66/Sec71 (Table 1; Table S6). Cellobiose and Avicel treatments also enhanced the abundances of some proteins involved in protein processing and export, including the signal peptidase complex subunit Spc2, the ER chaperone Hsp70-6, the protein disulfide isomerase Mpd1 as well as Sec62, Sec63, and Sec66/Sec71 (Liu et al., 2020).

| NCU no. | Annotation or domain | Phosphopeptide | PhosphoRS Site probability | CB versus NC Log2 fold change | p-value | AV versus NC Log2 fold change | p-value |
|---------|---------------------|----------------|---------------------------|-------------------------------|---------|-------------------------------|---------|
| NCU00169 | Translocation complex subunit (Sec63) | aEmkDEEE[pS] EPEEDSIAGIMNAAk | S645: 99.9; 0.44 0.018 | 0.99 0.001 |
| NCU02681 | Translocation complex subunit (Sec66/Sec71) | eLEGDEAGTG[pT] PVDTSSTVSSPSnk | T203: 99.8 −1.69 0.000 | −1.36 0.000 |
| NCU07746 | Translocation complex subunit (Sec72) | sISLAPSSGLSS[pS]VQTR | S33: 100.0 −0.35 0.055 | −0.90 0.000 |
| NCU09602 | Heat shock protein 70-1 (Hsp70-1) | kF[pS]DPEVQADmk | S77: 100.0 −0.78 0.001 | −0.71 0.016 |
| NCU02075 | Heat shock protein 70-2 (Hsp70-2) | sANITIS[pS]VGk | S482: 100.0 1.18 0.000 | 0.68 0.000 |

TABLE 1 (Continued)
glycosylation, and trafficking could not be assayed because they lacked homokaryotic mutants, indicating their essential roles in cell viability. In this study, we screened the mutants of some non-essential genes identified in our studies, including Sec66/Sec71, Spc2, and Hsp70-6 (Figure 4). Stk12, a serine-threonine protein kinase, was used as a positive control as its deletion increased lignocellulase production (Lin et al., 2019). We found that phosphorylation of Stk-12 on multiple sites was subject to regulation by cellobiose and Avicel (Table 1), and confirmed that deletion of stk-12 increased CMCase activities and secreted protein levels in the mutant cultures grown on Avicel (Figure 4). Cwh43, which is potentially involved in protein processing by transferring GPI anchor to nascent proteins, was used as another positive control as its deletion decreased lignocellulase production (Liu et al., 2020). We found that the abundance of Cwh43 was subject to regulation by cellobiose and Avicel (Liu et al., 2020), and confirmed that disruption of Cwh43 caused a decrease in CMCase activities and secreted protein levels in cultures grown on Avicel (Figure 4; Liu et al., 2020). In the screening assay, the level of proteins in the supernatant of mutant cultures grown on Avicel was measured to determine if the deletion mutations affect protein secretion under cellulolytic conditions. CMCase activity assay was used to examine if the deletion mutations affect the level of minor cellulolytic enzymes, particularly endoglucanases and LPMOs.

As shown in Figure 4, disruption of Sec66/Sec71 decreased CMCase activities and secreted protein levels, while disruption of Spc2 and Hsp70-6 increased CMCase activities and secreted protein levels, suggesting their potential roles in lignocellulase secretion. However, disruption of other genes that are also potentially involved in protein processing and export had no significant effect on CMCase activities and lignocellulase secretion, including protein transport genes: NCU04127 (Sss1, probable protein transport protein Sec61 subunit gamma) and NCU04724 (Lsb5, GO term: intracellular protein transport); protein processing genes: NCU00813 (Mpd1, disulfide isomerase), NCU02424 (DnaJ and TPR domain-containing protein, GO term: protein folding), and NCU11102 (Scj1, GO term: protein folding; Figure 4). Disruption of NCU02666 (NsdC, transcription factor) and NCU04826 (Hbc1, hyperbranching and cytoskeleton 1), which are involved in morphogenesis (Cary et al., 2012; Ellison et al., 2014), also had no effect on lignocellulase production. All the examined proteins showed either differential phosphorylation or differential abundance in response to cellobiose and Avicel (Liu et al., 2020; Table S2).

In fungal species, Sec66/Sec71 and Sec72 are bound to the Sec63 subunit of the Sec62–Sec63 complex (Itskanov et al., 2021), which associates with the Sec61 channel to mediate post-translational protein translocation (Wu et al., 2019). Sec66/Sec71 anchors Sec72 to the ER membrane while Sec72 provides a docking site for Hsp70 chaperones (Tripathi et al., 2017; Wu et al., 2019). LHS-1, the yeast ortholog of N. crassa Hsp70-6, is essential for protein translocation into the ER (Tyson & Stirling, 2000). The yeast ortholog of N. crassa Spc2, a noncatalytic subunit of the signal peptidase complex, interacts with the Sec61 channel in the ER (Antonin et al., 2000) and is involved in the suppression of signal peptide-independent protein translocation into...
TABLE 2 Proteins representative of different enriched GO terms with increased abundance but no significant change of mRNA levels of their encoding genes in response to Avicel cellulose

| Accession | Description | mRNA processing | | | rRNA processing and ribosome biogenesis | | | Protein processing and export | | |
|-----------|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cellobiose versus no carbon | Avicel versus no carbon | Avicel versus no carbon^a| Log2 fold change of protein abundance | Log2 fold change of protein abundance | Log2 fold change of mRNA abundance | Log2 fold change of mRNA abundance | Log2 fold change of mRNA abundance |
| **mRNA processing** | | | | | | | |
| NCU04606 | Pre-mRNA splicing factor | 1.47 | 0.000 | 0.63 | 0.037 | 0.39 | 0.65 |
| NCU07299 | Splicing factor 3a subunit 2 | 0.46 | 0.023 | 0.73 | 0.014 | 0.14 | 0.90 |
| **rRNA processing and ribosome biogenesis** | | | | | | | |
| NCU02604 | NET1-associated nuclear protein 1 (Nan1) | 1.40 | 0.000 | 2.01 | 0.000 | 0.25 | 0.44 |
| NCU00337 | Nucleolar complex-associated protein 3 | 0.59 | 0.023 | 1.07 | 0.004 | −0.12 | 0.83 |
| NCU06272 | rRNA biogenesis protein RRP5 | 0.45 | 0.030 | 0.71 | 0.018 | −0.89 | 1.00 |
| NCU03321 | Eukaryotic ribosome biogenesis protein 1 | 0.54 | 0.011 | 0.71 | 0.014 | −0.64 | 0.62 |
| **Protein processing and export** | | | | | | | |
| NCU05895 | Calcofluor white hypersensitive protein (Cwh43, GPI-anchoring) | 0.55 | 0.010 | 0.66 | 0.026 | −0.5 | 1.00 |
| NCU03503 | α-1,3/1,6-mannosyltransferase (Alg-2/Cot-5) | 0.60 | 0.006 | 0.63 | 0.034 | −0.25 | 1.00 |
| NCU04724 | VHS domain-containing protein (membrane trafficking) | 1.27 | 0.000 | 1.06 | 0.000 | −0.02 | 1.00 |
| NCU09121 | Vacuolar sorting protein | 0.48 | 0.022 | 0.84 | 0.003 | 0.19 | 1.00 |
| NCU02424 | DnaJ and TPR domain-containing protein (chaperone binding) | 1.18 | 0.000 | 1.15 | 0.000 | −1.12 | 0.38 |

^aTranscriptome data were derived from a previous study with similar experimental setup (Coradetti et al., 2013).
the ER (Hosomi et al., 2020). All these translocation-related proteins in *N. crassa* showed either differential phosphorylation or differential abundance upon exposure to cellobiose and Avicel. Specifically, Sec61, Sec63, Sec66/Sec71, Sec72, Hsp70-1, and Hsp70-2 showed differential phosphorylation (Table 1), while Hsp70-6, Sec62, Spc2 as well as Sec63 and Sec71 showed increased abundances (Liu et al., 2020), corroborating the idea that these proteins and their phosphorylations play important roles in lignocellulase translocation during cellulolytic responses. This is consistent with the deletion mutation analysis of Sec66/Sec71, Hsp70-6, and Spc2 (Figure 4).

### 3.5 Cellobiose-dependent cellular signaling for cellulolytic responses

Our quantitative proteomics analysis revealed that the abundance of Cdt1 (NCU00801) and Cdt2 (NCU08114), the cellodextrin transporters, and Sut12 (NCU05853), the transporter for cellobiose/cellulose oxidative product cellobionic acid, increased in response to both cellobiose and Avicel (Liu et al., 2020). Quantitative phosphoproteome profiling further revealed that Sut12 showed an increase in phosphorylation on T525 in response to cellobiose and Avicel (Table 1). Cdt1, Cdt2, and Sut12 may mediate cellobiose-dependent cellular signaling as previously proposed (Wu et al., 2013; Znameroski et al., 2012). This corroborates the observation that among 107 serine/threonine kinases and 30 protein phosphatases identified in *N. crassa* (Ghosh et al., 2014; Park et al., 2011), 37 and 11 were differentially phosphorylated, respectively, in response to cellobiose and/or Avicel (Tables S7 and S8). The differentially phosphorylated kinases and phosphatases include those involved in signal transduction mediated by cAMP/cGMP, small GTPase, MAPK, Ca²⁺, and polyphosphoinositol (Figure 2). A recent study suggests that G-protein signaling is required for cellulase production in *N. crassa* and positively regulates cellulase production via cAMP levels through adenylyl cyclase (CR1; Collier et al., 2020). We found that the effectors of the cAMP/cGMP signaling pathway, including CR1, PKAc1, and PRK2, were differentially phosphorylated in response to both cellobiose and Avicel (Table 1). Data from the STRING database showed that PKAc1 may interact with the protein kinase Stk12 (Figure S2), which has been shown to act as a repressor of cellulolytic gene expression (Figure 4; Lin et al., 2019). Stk12 is suggested to phosphorylate the downstream effector Igo1, which, in turn, activates cellulolytic gene expression by inhibiting the downstream target PP2A (Lin et al., 2019). Here, we found that cellobiose and Avicel induced...
differential phosphorylation of Stk12, Igo1, and the PP2A subunit Rrd2 (Table 1), indicating the importance of phosphorylation in regulating the signal transduction pathways during cellulolytic responses.

In addition to G-protein and cAMP pathways, MAP kinase pathways also play a role in regulating the production of cellulolytic enzymes in filamentous fungi (de Paula et al., 2018; Huberman et al., 2017; Schmoll, 2018; Wang et al., 2017). Differential phosphorylation was identified for the effectors of MAK-1 pathway (e.g., PKC-1, MIK-1, MEK-1, and MAK-1) and OS pathway (e.g., OS-1, RRG-1, OS-4, and OS-5; Table S7), further supporting the idea that post-translational modifications, particularly phosphorylation and dephosphorylation, play a critical role in signal perception, transmission, and subsequent cellular responses under cellulolytic conditions (Horta et al., 2019; Rodriguez-Iglesias & Schmoll, 2019; Schmoll, 2018; Xiong, Coradetti, et al., 2014). However, there is currently only limited insight into how protein kinases/phosphatases and their phosphorylation events are involved in cellulolytic responses in filamentous fungi (Cziferszky et al., 2002; Lin et al., 2019; Rodriguez-Iglesias & Schmoll, 2019; Wang et al., 2017).

4 CONCLUSION

In summary, we revealed a shared proteome and phosphoproteome response to cellobiose and Avicel cellulose, which corroborates the idea that cellobiose mediates the regulation of lignocellulase expression and secretion during cellulolytic conditions.
responses (Figure 5). In early adaptation to cellulose, the cellulolytic product cellobiose regulates the transcription of cellulolytic genes by modulating the phosphorylation state of the cellulolytic transcription factors, such as Clr1, Clr3, Cre1, and Vib1 (Table 1), and the abundance of Vib1 (Liu et al., 2020). Epigenetic control may also play a role in cellulolytic responses through the factors, such as Set1, Set2, Set4, and Set8 (Table 1). It was also noted that some proteins showed increased abundance but no significant change of mRNA levels (Figure 3), and they are potentially involved in post-transcriptional and post-translational regulation of early cellulolytic responses, including mRNA and rRNA processing and ribosome biogenesis (Table 2). At the translational level, cellobiose regulates the expression of cellulolytic enzymes by modulating the phosphorylation state of translational initiation factors, such as elf2b, elf4b, elf4e, elf4e3, elf5, and rli1 (Table 1). At the post-translational level, cellobiose induces differential expression and/or phosphorylation of the proteins involved in protein translocation across the ER (e.g., Hsp70-6, Spc2, Sec61, Sec62, Sec63, Sec66/Sec71, Sec72; Figure 4), protein processing in the ER (e.g., Cwh43, Pdi1, Grp78, Mpd1), and protein transport from the ER to the plasma membrane (e.g., Sec16, Erv29, Syl1). Lignocellulase production may further be controlled by the signal transduction pathways mediated by cAMP/cGMP, small GTPase, MAPK, Ca2+, and polyphosphoinositol (Figure 2). Altogether, the results offer a new insight into how cellulolytic products such as cellobiose mediate the regulation of lignocellulase expression and secretion and, furthermore, a potential strategy for strain engineering of filamentous fungi to improve the production of lignocellulases (Figure 5).

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AUTHOR CONTRIBUTIONS

S.C. designed research. B.X., L.W., Jinyu L., and Y.W. performed research. Y.M., X.L., Y.Z., P.D., S.G. analyzed data. S.C. wrote the paper with support from B.X., L.W., and Y.W. Shaolin Chen provided critical insights.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Johannes Liesche https://orcid.org/0000-0001-8753-4037
Shaolin Chen https://orcid.org/0000-0001-7607-8275

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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