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Original Research Article

# Investigating the cellular functions of β-Glucosidases for synthesis of lignocellulose-degrading enzymes in *Trichoderma reesei*

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ABSTRACT

β-glucosidases play an important role in the synthesis of cellulase in fungi, but their molecular functions and mechanisms remain unknown. We found that the 10 putative β-glucosidases investigated in *Trichoderma reesei* facilitate cellulase production, with *cel3j* being the most crucial. Transcriptional analysis revealed that the most affected biological processes in △*cel3j* strain were cellulase synthesis, ribosome biogenesis, and RNA polymerases. Moreover, CEL3J was unconventionally transported through the endoplasmic reticulum, bypassing the Golgi apparatus, whereas *cel3j* overexpression altered cellulase secretion from conventional to unconventional, likely owing to the activated unconventional protein secretion pathway (UPS), as indicated by the upregulation of genes related to UPS. The mTORC1-GRASP55 signaling axis may modulate the unconventional secretion of CEL3J and cellulase. The transcriptional levels of genes associated with DNA replication, the cell cycle, and meiosis were noticeably affected by overexpressing *cel3j*. These data give new clues for exploring the roles of β-glucosidases and the molecular mechanisms of their unconventional secretion in fungi.

## 1. Introduction

Plant biomass, an attractive alternative to fossil reserves, is the most sustainable and renewable carbon source on Earth and can be converted into environmentally friendly energy compounds and chemicals [1]. *Trichoderma reesei* is one of the most commonly used fungi for cellulase production. Cellulase is an enzyme mixture, mainly comprised of endoglucanase (EC 3.2.1.4), cellobiohydrolase (CBH; EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21), which act sequentially and synergistically to degrade biomass [1–5]. Among these three major components of cellulase, β-glucosidase is rate-limiting owning to its low secretive ability in *T. reesei*, which largely restricts the total efficiency of biomass degradation. Therefore, considerable efforts have been made to improve the protein secretion ability of β-glucosidase in *T. reesei* [1].

In total, 11 β-glucosidases have been predicted in the *T. reesei* genome. These are classified into glycoside hydrolase families 1 (*cel1a* and *cel1b*) and 3 (*cel3a, cel3b, cel3c, cel3d, cel3e, cel3f, cel3g, cel3h*, and *cel3j*) on the basis of their sequence identities and structural similarities [6]. β-glucosidases play an important role in the induction and production of cellulase. CEL3A, the major extracellular β–glucosidase, functions in rapid cellulase induction in *T. reesei* [7,8], whose overexpression can effectively enhance cellulase production [9–12]. The absence of *cel1b* and *cel1a* delays or even abolishes the expression of the major cellulase gene *cbh1* in cellulose or lactose, indicating their involvement in rapid cellulase induction [13,14]. In contrast, the deletion of *cel1b* does not noticeably affect cellulase production in cellulose, cellobiose, or lactose, whereas overexpression of *cel1b* dramatically decreases cellulase synthesis, likely due to dysfunction of the cellular transport process and the endoplasmic reticulum (ER) [15]. Comprehensive cellular distribution and secretion analysis identifies eight extracellular (CEL1A, CEL3A, CEL3B, CEL3E, CEL3F, CEL3G, CEL3H, and CEL3J) and three intracellular β-glucosidases (CEL1B, CEL3C, and CEL3D) [15–17]. The extracellular β-glucosidases CEL3A, CEL3B, CEL3E, CEL3F, CEL3H, and CEL3G are secreted through a tip-directed conventional secretion pathway, and CEL1A via a vacuole-mediated pathway with no involvement of a signal peptide. In particular, the leaderless protein CEL3J is secreted via an unconventional protein pathway, which raised our interest in studying the molecular functions and secretive mechanisms of CEL3J.

Generally, proteins can reach the extracellular space or plasma membrane by two secretion pathways. The canonical ER-to-Golgi secretory pathway, which has been known for decades, transports the majority of secretory proteins [18,19]. These proteins usually carry a signal peptide

*Abbreviations:* CBH, Cellobiohydrolase; ER, Endoplasmic reticulum; BFA, Brefeldin A; Endo H, Endoglycosidase H; UPS, Uprotein secretion pathway; AMT, *A. tumefaciens*-mediated transformation; GFP, Green fluorescence protein.

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to direct their translocation into the ER lumen for protein folding, from where they reach the Golgi apparatus via COPII-coated vesicles for further protein modification and the plasma membrane for protein release [16]. However, over the past 20 years, a substantial number of proteins that are not, or are only partially, dependent on the classical secretion pathway, which is collectively termed "unconventional protein secretion" (UPS) have been identified [19,20]. Four different UPS pathways have been described: Type I, self-sustained protein translocation across the plasma membrane; type II, ATP-binding cassette transporter–based secretion; Type III, secretion by membrane-bound organelles such as autophagosomes; and Type IV, Golgi bypass pathway to the plasma membrane [21,22]. Leaderless proteins are translocated across the plasma membrane by Type I, II, and III UPS, whereas proteins with a signal peptide or transmembrane domain depend on Type IV UPS. Proteins that traffic through the Golgi bypass pathway can be identified by two main methods: Brefeldin A (BFA) insensitivity and Endoglycosidase H (Endo H) sensitivity [23,24]. BFA, a fungal toxin, acts on guanine nucleotide exchange factors and interferes with the recruitment of Arf1, an enzyme responsible for the formation of COPI-coated vesicles, blocking anterograde ER-to-Golgi transport [23]. Therefore, proteins that reach the plasma membrane that are BFA-insensitive are Golgi-independent. Protein modification occurs in the ER by glycosylation with a high-mannose-containing, *N*-linked oligosaccharide core [25] and then in the Golgi apparatus by trimming and adding sugars to this core, generating complex *N*-glycans [26]. Therefore, if a protein is sensitive to Endo H, an enzyme that only cleaves high-mannose and some hybrid types of *N*-linked carbohydrates, its secretion is thought to bypass the Golgi apparatus. Many types of cargo have been identified to reach the plasma membrane independent of the Golgi apparatus, including CFTR [20,24,27], $\alpha$PS1 [28,29], $\alpha$PS2 [30], CD45 [31], Smoothened [32], Mpl [33], pendrin [34]. Golgi reassembly stacking proteins (GRASPs) [22,24,29,35], heat shock proteins, cochaperones [36,37], TMED10 [38], and TORC1 [22] have been implicated in the Golgi bypass pathway [24]. However, the molecular mechanisms of the Golgi bypass pathway remain unclear.

In this study, we investigated the impact of the individual deletion of 10 $\beta$-glucosidases on cellulase production, growth, and mycelium morphology using different carbon sources; we found that the absence of each $\beta$-glucosidase compromised cellulase synthesis, and that *cel3j* deletion caused the largest decrease in cellulase production. Transcriptional analysis was performed to explore the molecular mechanisms underlying the severe inhibition in the *cel3j* knockout. Furthermore, the unconventional secretion of CEL3J was found to occur through the Golgi-bypassing pathway, while cellulase was transported via the unconventional protein pathway in the *cel3j*-overexpressing strain Rcel3J, as opposed to via the conventional secretion mode as in the parental strain RUT-C30. The mTORC1-GRASP55 signaling axis may participate in the unconventional secretion of both CEL3J and cellulase. Overexpression of *cel3j* upregulates the transcriptional abundance of genes associated with DNA replication, DNA repair, the cell cycle, and meiosis. Overall, these findings enhance our understanding on $\beta$-glucosidases and the unconventional secretion pathway in filamentous fungi.

## 2. Materials and methods

### 2.1. Microbial strains, plasmids, and cultivation conditions

Microbial strains (*Escherichia coli* DH5$\alpha$, *Agrobacterium tumefaciens* AGL-1, *T. reesei* RUT-C30, and *T. reesei* KU70), their culture conditions, and plasmids pXBthg and pDht/sk were adapted from our previous studies [15,39-41].

### 2.2. Construction of the $\beta$-glucosidase deletion strain ($\triangle$BGL)

The upstream and downstream sequences (approximately 1500 bp) of the nine individual $\beta$-glucosidases were separately amplified by PCR using the *T. reesei* KU70 genome as a template, and ligated into the plasmid pXBthg at *Xho*I and at *Bam*HI using the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China), leading to the generation of different pXBthg-$\triangle$BGL ($\beta$-glucosidases = *cel1a, cel3b, cel3c, cel3d, cel3e, cel3f, cel3g, cel3h*, or *cel3j*) plasmids. The resulting plasmids pXBthg-$\triangle$BGL were individually transformed into *T. reesei* KU70 via the *A. tumefaciens*-mediated transformation (AMT) method using hygromycin B as a marker [42], yielding the corresponding $\triangle$BGL. The primers used are listed in Table S1, and gene deletions in the recombinant strains were verified by PCR (Figure S1) and sequencing.

### 2.3. Shake flask cultivation

Five percent (v/v, $10^7$ /mL) conidia of *T. reesei* were seeded into 10 mL sabouraud dextrose broth (Glucose, 40 g/L; Tryptone, 10 g/L; Yeast extract, 10 g/L) at 28 ˚C and 200 rpm for 2 days. Then, 5 mL pre-grown mycelia was transferred into 50 mL TMM culture medium (Tryptone, 0.75 g/L; Yeast extract, 0.25 g/L; Urea, 1.00 g/L; $(NH_4)_2SO_4$, 4.00 g/L; $KH_2PO_4$, 6.59 g/L; Maleic acid, 11.6 g/L; $FeSO_4 * 7H_2O$, 0.005 g/L; $MnSO_4 * H_2O$, 0.0016 g/L; $ZnSO_4 * 7H_2O$, 0.0014 g/L; $CoCl_2 * 6H_2O$, 0.002 g/L; $MgSO_4$, 0.60 g/L; $CaCl_2$, 0.60 g/L; Tween 80, 0.186 ml/L) (pH 6) plus 2% cellulose or other carbon sources as indicated and incubated at 28 ˚C for 7 days. For the BFA or rapamycin experiments, 5.0 mL cultures, which were grown in TMM plus 2% cellulose for 4 days, were transferred into the same fresh TMM plus 2% cellulose supplemented with BFA (10 $\mu$g/mL) or rapamycin (0.01, 1, and 10 $\mu$mol/L) and cultured for 7 days. Samples were collected at different time points for the (hemi)cellulase activity assay, confocal microscopy, RNA-seq, and qRT-PCR analyses.

### 2.4. Analysis methods

The samples of *T. reesei* culture were centrifuged at 8000 rcf for 30 min to collect the supernatants for the (hemi)cellulase activity assay, as described in our previous studies [9,17,39,40]. The mycelia of *T. reesei* were observed under a confocal SP8 microscope (Leica, Mannheim, Germany) with a 100 × oil immersion objective. The spores were counted using a hemocytometer under an SP8 confocal microscope with a 20 × oil immersion objective. The fluorescence intensities of the supernatant were measured at excitation and emission wavelengths of 540/635 nm, respectively, using a fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan).

### 2.5. Endo H sensitivity assay

Gene *cel3j* was amplified from *T. reesei* RUT-C30 with His-tag sequence fused to its 3′-end, and ligated into the plasmid pDht/sk at *Xba*I using the ClonExpress One Step Cloning Kit (Vazyme, Nanjing, China). The resulting vector was subsequently transformed into *T. reesei* RUT-C30 using the AMT method with hygromycin B as the marker, resulting in generation of the strain OEcel3J. Strain OEcel3J was grown in TMM medium containing 2% cellulose at 28 ˚C for 7 days, and the supernatants of the culture were used to purify CEL3J using the Ni-NTA 6FF Sefinose (TM) Resin kit (Sangon Biotech, Shanghai, China). Purified CEL3J was concentrated and desalted in an Amicon-Ultra-15 centrifuge tube (Millipore Corporation, Billerica, MA, USA) with a molecular weight cutoff of 30 kDa. For digestion of CEL3J by Endo H (New England Biolabs, Ipswich, Massachusetts, United States), the purified CEL3J was first denatured by adding Denaturing Buffer (New England Biolabs, Ipswich, Massachusetts, United States) at 100 ˚C for 10 min. Then, the reaction was treated with Endo H and further incubated at 37 ˚C for 2 h. The (non)-treated CEL3J cells were detected by western blotting with an Anti-His6 antibody (Bioss, Beijing, China).

### 2.6. CEL3J colocalization with ER

The gene encoding geneticin (G418) was obtained by PCR using plasmid lcNG as the template [43] and ligated into pDht/sk after digestion with *Bam*HI and *Spe*I to generate the plasmid pDhG418. The gene encoding green fluorescence protein (GFP) was amplified from the plasmid EGFP-xyr1, which was provided by Professor Weixin Zhang of Shandong University, with its N-terminus and C-terminus fused with the ER-targeting peptide and the ER-retention motif (HDEL) of disulfide isomerase (PDI1), respectively, and ligated into the plasmid pDhG418, resulting in generation of the plasmid pEGFP-HDEL. The resulting vector pEGFP, was subsequently transformed into *T. reesei* Rcel3J using the AMT method with hygromycin B and G418 as markers [42], yielding the strain Rcel3J-GFP. The red fluorescent protein, DsRed, was detected at 570–700 nm with an excitation wavelength of 552 nm. The green fluorescence protein GFP was imaged at 500–550 nm with an excitation wavelength of 488 nm.

### 2.7. RNA-seq and qRT-PCR analysis

*T. reesei* samples, which were cultured in TMM + 2% cellulose, were collected at 72 h for △cel3j and KU70, and 120 h for Rcel3J and RUT-C30. RNA-sequencing was carried out in duplicate using Novogene (Beijing, China) following the standard analysis method. RNA extraction and reverse transcription were performed as previously described [15,39,41]. Quantitative PCR was performed using the TB Green Premix Ex Taq II (TaKaRa Bio, Tokyo, Japan), and relative mRNA levels were normalized to the housekeeping gene *sar1* [44]. All primers used in this study are listed in Table S1.

### 2.8. Statistical analysis

Some of the statistical data presented are the mean ± SD from at least three independent experiments. Two-tailed Student's *t*-test was performed to analyze the difference between two groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

## 3. Results

### 3.1. Cellulase production was compromised by the single deletion of β-glucosidases

To investigate the role of the 11 putative β-glucosidases on cellulase production in *T. reesei*, we individually deleted nine β-glucosidase genes (*cel1a, cel3b, cel3c, cel3d, cel3e, cel3f, cel3g, cel3h*, and *cel3j*) in *T. reesei* KU70 to obtain the deletion strains △cel1a, △cel3b, △cel3c, △cel3d, △cel3e, △cel3f, △cel3g, △cel3h, and △cel3j, respectively (Figure S1). The *cel1b*-knockout strain △cel1b was constructed in our previous study [15], and the severe inhibitory effect of *cel3a* deletion on the synthesis of cellulase by fungi has been well studied in our previous research [8]. When cultured in TMM + 2% cellulose and compared to the parental strain KU70 (Fig. 1A), FPase activity was markedly decreased in △cel1b, △cel3b, △cel3e, and △cel3j strains by 48.5%, 42.1%, 32.1%, and 77.1% respectively, but remained almost unchanged in △cel1a, △cel3c, and △cel3d strains. All the recombinant strains showed a notably decrease in pNPCase and pNPGase activities. CMCase activity was not noticeably affected in all knockout strains, except for △cel3c and △cel3j. pNPXase activity was markedly reduced in △cel3b, △cel3e, △cel3f, △cel3g, and △cel3j strains. Secreted protein levels decreased noticeably in strains △cel1a, △cel3b, △cel3c, △cel3e, △cel3h, and △cel3j. The absence of each β-glucosidase compromised (hemi)cellulase production to varying degrees and with different patterns. In particular, the deletion of *cel3j* led to the largest decrease in (hemi)cellulase activity.

When using 2% lactose as the sole carbon source, cellulase production was also affected by the deletion of different β-glucosidases to varying degrees, of which the most affected was pNPCase activity (Fig. 1B). pNPXase activity was decreased in the △cel1a, △cel1b, △cel3c, △cel3d, △cel3e, △cel3g, and △cel3j strains, but only slightly altered in △cel3b, △cel3f, or △cel3h strains. Secreted protein levels were not impaired in △cel3e and △cel3f strains, whereas they were decreased in the other recombinant strains. Similar to what was observed on cellulose, the △cel3j strain was inhibited the most severely regarding (hemi)cellulase activities, including FPase, pNPCase, CMCase, pNPGase, pNPXase, and protein secretion. These results suggest that all 10 β-glucosidases play positive and likely varied roles in (hemi)cellulase production.

Cellulase production on both cellulose and lactose was dramatically affected by the deletion of *cel3j*, indicating that the cellulase-inhibiting effect of the *cel3j* knockout might be independent of the carbon source. To further confirm this, we measured the cellulase activity of Δcel3j strain cultured on other carbon sources. As shown in Fig. 1C, the (hemi)cellulase activities of Δcel3j strain severely declined when cultured on 2% glucose, galactose, or glycerol, while protein secretion remained constant in Δcel3j strain. Overall, these findings imply that *cel3j* plays a crucial role in cellulase production, regardless of the carbon source.

### 3.2. Influence of β-glucosidase deletion on growth, sporulation, and morphology of T. reesei

The effect of β-glucosidase deletion on cell growth, spore ability, and morphology of *T. reesei* under cellulase-producing conditions was investigated. The colony diameters of all the △BGL tested, with the exception of △cel3b strain, were not significantly different compared to those of the parental strain KU70 (Fig. 2A). Their spore levels were similar to those of KU70, with the exception of those of △cel1a strain, which exhibited a moderate reduction in spore levels (Fig. 2B). Moreover, confocal imaging of all the mutant strains showed that their mycelial morphologies were not significantly altered (Fig. 2C). Taken together, the individual deletion of β-glucosidases in *T. reesei* did not markedly influence the phenotype of *T. reesei*, including cell growth, spore ability, and morphology.

### 3.3. Transcriptional analysis of strain △cel3j

Given that △cel3j strain had the greatest effect on cellulase activity, RNA-seq analysis was performed to understand how *cel3j* deletion influenced *T. reesei* at the transcriptional level. Genes were considered to be differentially expressed (DEG) between △cel3j and KU70 strains cultivated in TMM + 2% cellulose for 72 h when the average reads of the corresponding transcripts differed with $|\log_2 \text{Ratio}| \geq 1$ and $p$ value $\leq 0.05$. Based on this analysis, 2 435 of the 9 595 identified genes were DEGs, among which 1186 were upregulated and 1249 were downregulated (Table S2). The most enriched biological process was carbohydrate metabolism (Fig. 3). Unexpectedly, "ribosome biogenesis," "ribonucleoprotein complex biogenesis," "rRNA processing," and "ncRNA processing" were also enriched and all genes in these processes, except two, were upregulated (Table S3), indicating that *cel3j* deletion promoted ribosome biogenesis and rRNA process at the transcriptional level. Consistently, the categories "ribosome biogenesis in eukaryotes" and "RNA polymerase" in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were also enriched (Fig. 3). All 15 DEGs in "RNA polymerase" were upregulated (Table S3).

Transcriptome analysis revealed 51 DEGs related to cellulase synthesis, including 6 upregulated and 45 downregulated genes (Table S4). All DEGs encoding (hemi)cellulase, except two genes, were decreased at the mRNA level, in agreement with the significantly decreased cellulase activities of △cel3j strain. The non-enzymatic cellulose-attacking enzymes swollenin [45], Cip1, and Cip2 [46], which act in synergy with (hemi)cellulases to enhance the hydrolytic efficiency of cellulose, were

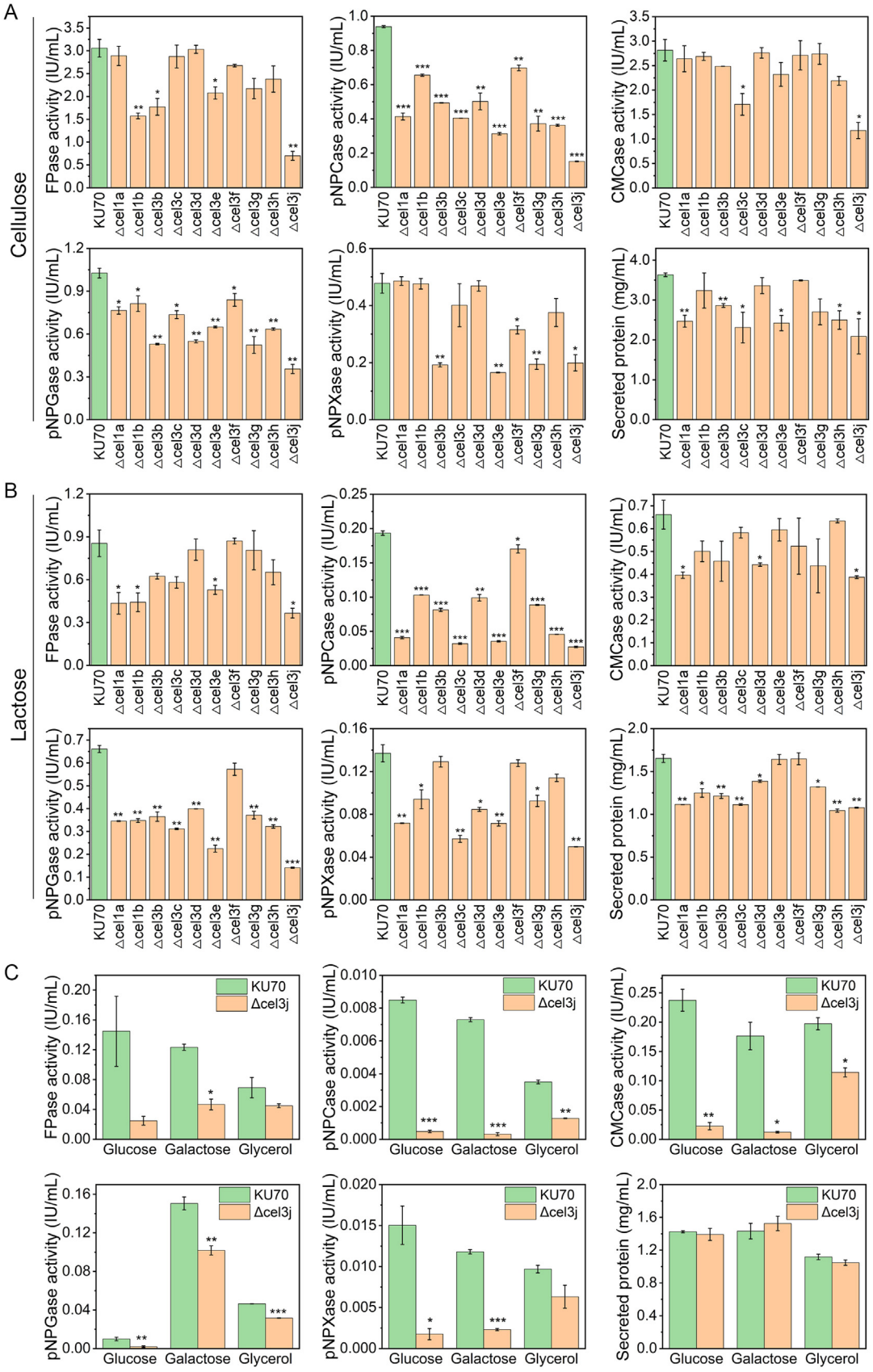


**Fig. 1.** Cellulase activities and protein secretion for *T. reesei* KU70 and △cel1a, △cel1b, △cel3b, △cel3c, △cel3d, △cel3e, △cel3f, △cel3g, △cel3h, and △cel3j recombinant strains cultured in TMM using different carbon sources. (A) 2% cellulose for 120 h; (B) 2% lactose for 72 h; (C) 2% glucose, 2% galactose, and 2% glycerol for 120 h. Data are represented as the mean of three independent experiments, and error bars denote the standard deviations. Asterisks indicate significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) as assessed by the Student's *t*-test.

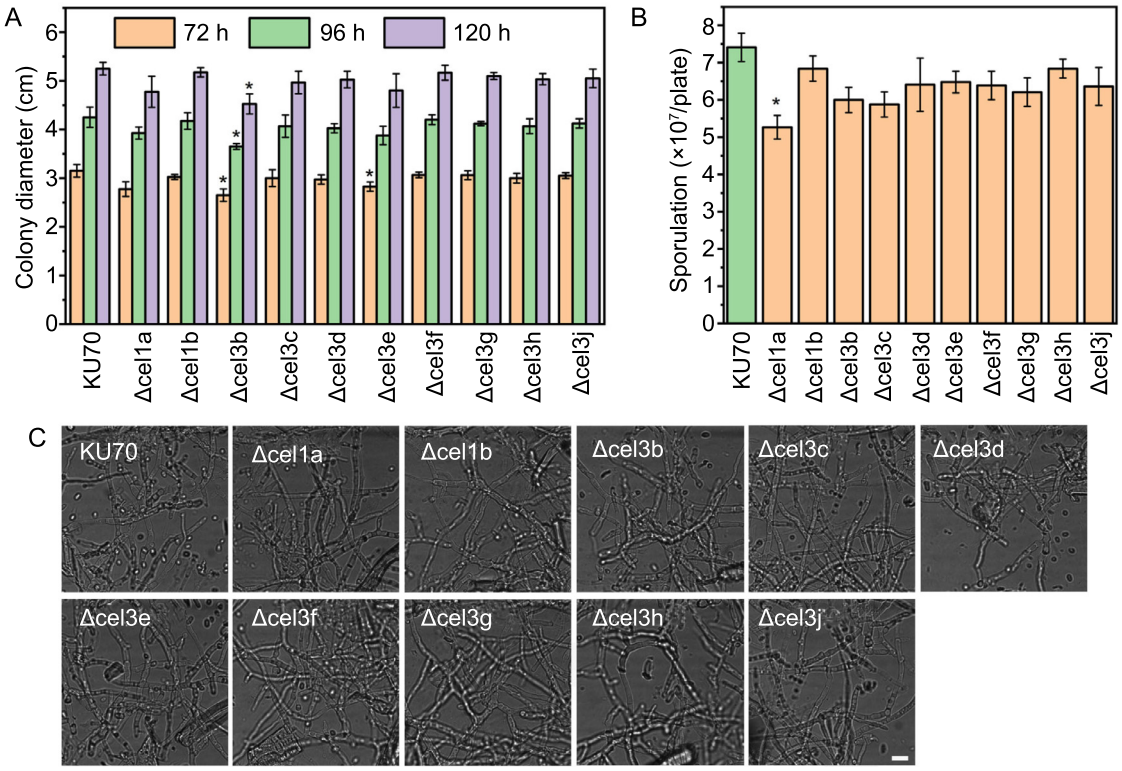


**Fig. 2.** (A) The colony diameter, **(B)** sporulation ability, and **(C)** morphology of *T. reesei* KU70, △cel1a, △cel1b, △cel3b, △cel3c, △cel3d, △cel3e, △cel3f, △cel3g, △cel3h, and △cel3j strains. All strains were cultured on TMM liquid or agar plates with 2% cellulose. The spores were counted at 120 h and cell morphology observation was performed at 72 h using a confocal microscope. Data are represented as the mean of three independent experiments, and error bars denote the standard deviations. Asterisks indicate significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) as assessed by the Student's *t*-test. Scale bar = 10 $\mu$m.

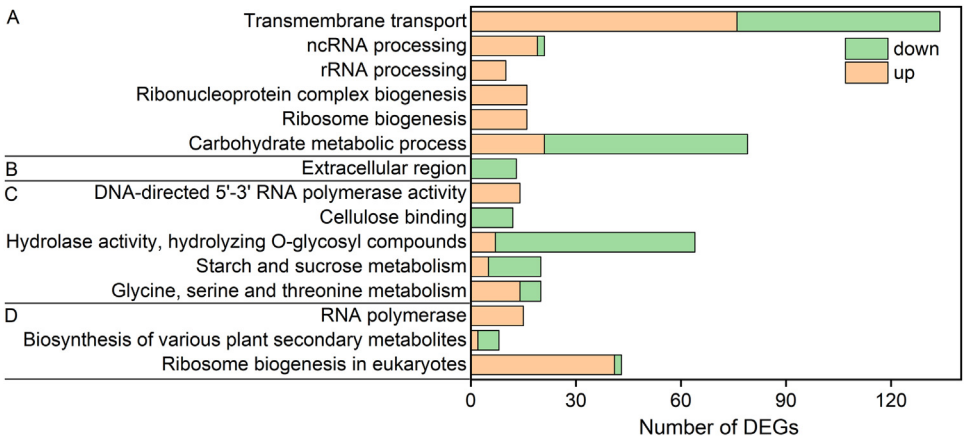


**Fig. 3.** Gene ontology (GO) functional enrichment (A–C) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment (D) analyses of DEGs. **(A)** The most enriched biological processes; **(B)** The most enriched cellular components; **(C)** The most enriched molecular functions; **(D)** The most enriched KEGG pathways. The y-axis represents the names of the most enriched GOs or pathways that belong to different ontologies. Gene Ratio: the number of DEGs in a specified GO term/the number of the total DEGs in all GO terms; Gene Number: the number of DEGs within a specified GO term.

significantly downregulated. In addition, nine transcription factors involved in cellulase production were identified as DEGs. Among them, the transcription activator Xyr1 [47], Azf1 [48], and vib1 [49,50], mitogen-activated protein kinase Tmk2 [51], and protein disulfide isomerase PDI1 [1] were downregulated, while the sugar transporter Stp1 [52,53], nuclear importer KAP8 [54], and transcription regulators VEL1 [55] and ctf1 [56] were upregulated.

### *3.4. CEL3J is secreted through the Golgi bypass pathway, overexpression of which activated the unconventional secretion of cellulase-*

Our previous study indicated that the protein CEL3J is likely secreted via an unconventional protein pathway bypassing the ER and Golgi [16]. To confirm that the secretion of CEL3J bypasses the Golgi apparatus, the sensitivity of extracellular CEL3J to Endo H was measured. Since Endo H can only cleave high mannose and some hybrid types of *N*-linked carbohydrates produced in the ER, but cannot cleave complex *N*-glycans generated in the Golgi apparatus, proteins that were sensitive to Endo H were considered to bypass the Golgi [24]. The gene *cel3j*, with its His-tag fused to the C-terminus of the encoding protein, was overexpressed in RUT-C30, yielding the recombinant strain OEcel3J for purifying CEL3J-Hisby nickel-affinity chromatography. Western blot analysis showed that incubation of purified CEL3J cells with Endo H led to a lower band intensity than that of the untreated cells (Fig. 4A). This demonstrated that CEL3J was Endo H-sensitive, strongly supporting our hypothesis that CEL3J was not transported through the Golgi apparatus, as indicated by the insensitivity of CEL3J secretion to BFA in our previous study [16]. This also indicated that CEL3J likely undergoes

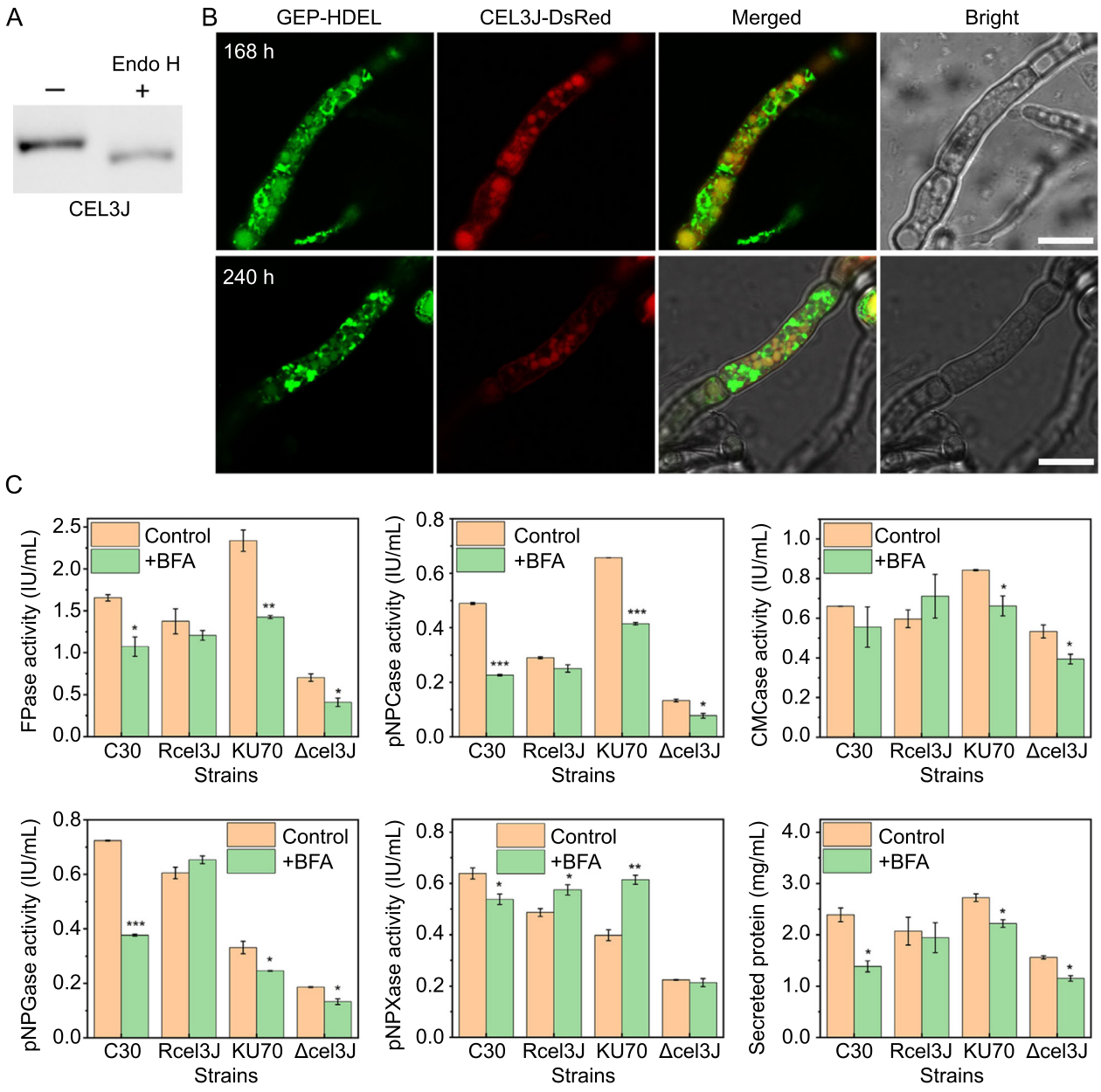


**Fig. 4.** (A) Endo H sensitivity assay of CEL3J. Western blot was performed using an Anti-His antibody to detect CEL3J-His, which was purified from the supernatant of OEcel3J and treated with Endo H (+). The untreated protein CEL3J-His (-) was used as a control. (B) Confocal images of the strain Rcel3J-GFP. Rcel3J-GFP was cultured on TMM + 2% cellulose for 168 h and 240 h. Scale bar = 10 $\mu$m. **(C)** The impact of BFA on cellulase activities and protein secretion of *T. reesei* RUT-C30, Rcel3J, KU70, and △cel3j at 120 h on TMM + 2% cellulose. Data are represented as the mean of three independent experiments, and error bars denote the standard deviations. Asterisks indicate significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) as assessed by the Student's *t*-test.

coreglycosylation in the ER, which is in conflict with our previous finding that CEL3J did not enter the ER, as indicated by the lack of overlap between CEL3J tagged with red fluorescent protein and green-emitting ER-tracker [16]. To determine whether CEL3J was distributed in the ER, the degree of colocalization of CEL3J with the ER was determined. Instead of using an ER tracker, the N-and C-termini ofGFPwere fused with the ER-targeting peptide and the ER-retention motif (HDEL) of the ER-resident protein PDI1, respectively, as markers for visualization of the ER [57,58]. This fusion protein marker was named GFP-HDEL. The plasmid pEGFP-HDEL (Figure S2) for the expression of GFP-HDEL was transformed into the Rcel3J strain, which expressed CEL3J labeled with the red fluorescent protein DsRed, yielding the recombinant strain Rcel3J-GFP. In Rcel3J-GFP strain, a large area of yellow fluorescence was observed owing to the overlap of green fluorescence with red fluorescence (Fig. 4B), demonstrating that CEL3J was localized in the ER. These findings demonstrate that CEL3J is transported via the ER, bypassing the Golgi (i.e., the type IV unconventional secretion pathway).

Our previous study showed that the levels of secreted CEL3J and the pNPGase activity of Rcel3J strain were not sensitive to BFA, a protein inhibitor of vesicle trafficking between the ER and Golgi in fungi [16]. Therefore, we investigated whether the secretion of all cellulase components in Rcel3J was BFA-resistant. The cellulase activities of RUT-C30 and Rcel3J cells treated with and without BFA were measured (Fig. 4C). The addition of BFA partially inhibited the FPase, pNPCase, and pNPGase activities and total protein secretion of RUT-C30, but did not affect those of Rcel3J. Interestingly, the inhibition of CMCase activ-

ity in RUT-C30 was not as significant as that of other cellulase components, which was consistent with a previous finding that CMCase activity in RCMC strain overexpressing CMC was not very sensitive to BFA [17]. The insensitivity of cellulase components and protein secretion in Rcel3J to BFA was not colony-specific, as another three random selected transformants displayed the same results as Rcel3J (Figure S3). Furthermore, SDS-PAGE analysis showed that the protein secretion level of CEL7A (the main cellobiohydrolase) in Rcel3J did not change in the presence of BFA (Figure S4), in agreement with the unchanged pNPCase activity (Fig. 4C), while in the RUT-C30 it decreased with BFA treatment (Figure S4). Contrary to that of Rcel3J, the cellulase secretion of the △cel3j strain was sensitive to BFA, as shown by decreases of 41.8%, 41.7%, 26.1%, and 28.6% in FPase, pNPCase, CMCase, and pNPGase activities, respectively, of BFA-treated △cel3j in comparison with those of untreated △cel3j. In the presence of BFA, hemicellulase pNPXase activity was slightly increased in Rcel3J and unchanged in △cel3j. These findings suggest that cellulase is mainly secreted through an unconventional protein secretion pathway in strain Rcel3J, differing from in RUT-C30, where cellulase is secreted mainly via the conventional protein secretion pathway [17]. Overexpression of the unconventional secretion protein CEL3J altered cellulase secretion in *T. reesei* from conventional to unconventional. It is possible that *cel3j* overexpression activates an unconventional secretion pathway that is harnessed for cellulase secretion, endowing *T. reesei* with resistance to BFA.

### 3.5. The mTORC1-GRASP55 signal axis may be involved in the unconventional secretion of CEL3J and cellulase

Recently, mTORC1-GRASP55 was revealed as a key signaling pathway for unconventional protein secretion, through which numerous proteins are transported to the extracellular space under cellular stress [22]. Under normal conditions, GRASP55 is phosphorylated by active mTORC1 and is localized in the Golgi apparatus. In response to stress signals, such as the inhibition of mTORC1 activity by rapamycin, GRASP55 relocates to autophagosomes and multivesicular bodies, triggering the unconventional secretion of certain proteins. Therefore, we investigated whether mTORC1-GRASP55 is involved in the unconventional secretion of CEL3J cells. Rapamycin was added to the TMM +2% cellulose medium, and the fluorescence intensity of the culture supernatant of Rcel3J was measured. Fluorescence intensity was stronger in the presence of 0.01 $\mu$mol/L rapamycin (Fig. 5A). This phenomenon was more evident when Rcel3J cells were treated with 1 or 10 $\mu$mol/L rapamycin (Fig. 5A). At 168 h, the fluorescence intensity of Rcel3J with 1 or 10 $\mu$mol/L rapamycin was 2.04- and 2.15-fold, respectively, of that without rapamycin. These results demonstrate that rapamycin treatment markedly promotes CEL3J secretion in Rcel3J cells. Moreover, the transcriptional levels of typical genes related to the mTORC1-GRASP55 signaling axis for coordinating unconventional protein secretion in Rcel3J were analyzed by RNA-seq and compared to those in RUT-C30 (Fig. 5B). The TOR, TSC2, and GRASP55 genes in Rcel3J showed obvious increases of 60.6, 46.3, and 44.2%, respectively. It is possible that the mTORC1-GRASP55 signaling pathway participates in the unconventional secretion of CEL3J, which requires further investigation.

In addition, the secretion of other cellulase components in Rcel3J was not inhibited by rapamycin, as shown by the FPase, pNPCase, CMCase, and pNPGase activities, and the total protein secretion levels were the same as or higher than those untreated with rapamycin during the entire fermentation process (Fig. 5C). Interestingly, the pNPXase activity of Rcel3J was inhibited at the early stage (24 h) and did not recover at the late stage (168 h) in the presence of rapamycin, showing the same pattern as that of RUT-C30. This result differs from that of RUT-C30, where cellulase activity was severely impaired by 10 $\mu$mol/L rapamycin during the early fermentation stage [17]. Overexpression of *cel3j* renders cellulase secretion from *T. reesei* resistant to a higher concentration of rapamycin, indicating that the mTORC1-GRASP55 signaling axis may also be involved in the unconventional secretion of cellulase in addition to CEL3J, but not hemicellulase.

**Table 1**
Transcriptional levels of UPS genes in *T. reesei* Rcel3J and RUT-C30.

| Name | Protein ID | $\log_2$FC | *p* value | Rcel3J/RUT-C30 |
|---|---|---|---|---|
| HSP90 | M419DRAFT_102,206 | 1.067 | 3.19E–55 | 2.095 |
| HSP70 | M419DRAFT_121,926 | 0.830 | 7.44E–36 | 1.777 |
| TOR | M419DRAFT_24,714 | 0.684 | 1.70E–12 | 1.606 |
| TSC2 | M419DRAFT_68,949 | 0.549 | 4.32E–06 | 1.463 |
| GRASP55 | M419DRAFT_7104 | 0.528 | 0.0009 | 1.442 |
| *Vps23* | M419DRAFT_82,444 | 0.462 | 0.0381 | 1.378 |
| TMED10 | M419DRAFT_124,198 | 0.425 | 1.26E–06 | 1.343 |
| *Vps25* | M419DRAFT_83,120 | 0.338 | 0.1842 | 1.264 |
| DNAJC14 | M419DRAFT_135,863 | 0.310 | 0.0017 | 1.240 |
| RAB8A | M419DRAFT_75,875 | 0.202 | 0.0910 | 1.150 |
| *Sec5* | M419DRAFT_32,578 | 0.173 | 0.1989 | 1.127 |
| *Exo70* | M419DRAFT_5475 | 0.066 | 0.7234 | 1.047 |
| *IRE1α* | M419DRAFT_6675 | 0.050 | 0.3990 | 1.035 |
| *Snf7* | M419DRAFT_38,233 | 0.032 | 0.6994 | 1.022 |
| *Sso1* | M419DRAFT_139,928 | 0.028 | 0.8259 | 1.020 |
| *Atg8* | M419DRAFT_120,275 | –0.157 | 0.236 | 0.897 |

### 3.6. Transcriptional patterns of strain RCEL3J

RNA-seq analysis was performed to study how *cel3j* overexpression influenced *T. reesei* RUT-C30 at the transcriptional level. Of the 9 522 genes identified in the genome of *T. reesei*, 1205 were DEG in the Rcel3J strain. Among these, 554 were upregulated, and 651 were downregulated (Table S5). Gene ontology (GO) functional enrichment analysis of these DEGs showed that the most enriched molecular function was mainly related to “hydrolase activity acting on glycosyl bonds” (Fig. 6), which comprised 36 DEGs. Among them, nine DEGs were upregulated and 27 were downregulated (Table S5). For the enriched cellular components, 14 DEGs were under the “extracellular region” category, demonstrating that overexpression of *cel3j* impacts extracellular enzymes (Fig. 6). The enriched biological processes included “DNA replication,” “DNA metabolic process,” “Carbohydrate metabolic process,” and “Oxidation-reduction process.” In line with the enriched biological processes, the most enriched KEGG pathways were “DNA replication”, “cell cycle”, “meiosis”, “mismatch repair”, “starch and sucrose metabolism”, and “nucleotide excision repair” (Fig. 6). Specifically, 22 DEGs involved in the enriched KEGG pathway “DNA replication,” including the Mini-Chromosome Maintenance (MCM) complex Mcm2–Mcm7, DNA polymerase $\alpha$-primase, $\delta$ and $\varepsilon$ complex, and DNA ligase Lig1 (Figure S5), 12 DEGs involved in “Mismatch repair,” 12 of 13 DEGs involved in “Nucleotide excision repair,” 33 DEGs involved in “Cell cycle,” and 24 of 26 DEGs involved in “Meiosis”, were upregulated (Table S6). These results demonstrated that overexpression of *cel3j* greatly influenced DNA biosynthesis and repair, the cell cycle, and meiosis, possibly through evolutionarily conserved TOR, a central controller of cell growth [59–61].

Many proteins involved in unconventional protein secretion have been identified. For example, GRASP55 has been implicated in the UPS pathway of proteins including Acb1, IL-1$\beta$, Sod1, $\alpha$PS1, CFTR, and Mpl [22,24,35,62,63]. TMED10 mediates the translocation of UPS cargo into vesicles, which is enhanced by HSP90s [38]. The TSC-mTORC1 signaling axis regulates the phosphorylation and relocalization of GRASP55 under stress, triggering the UPS of selected cargo [22]. Vps23, Vps25, and Snf7 are required for Golgi bypass of Acb1 secretion [63]. DNAJC14 and IRE1$\alpha$ are involved in H723R-pendrin secretion [37]. The cytoplasmic effectors of the rice blast fungus *Magnaporthe oryzae* require the exocyst components Exo70 and Sec5 for efficient secretion [64]. Atg8 has also been implicated in unconventional ΔF508-CFTR trafficking [35]. The transcript levels of these genes in *T. reesei* Rcel3J and RUT-C30 at 120 h, as determined by RNA sequencing, are listed in Table 1. Although only HSP90 belonged to the DEG based on our strict standard, the ex-

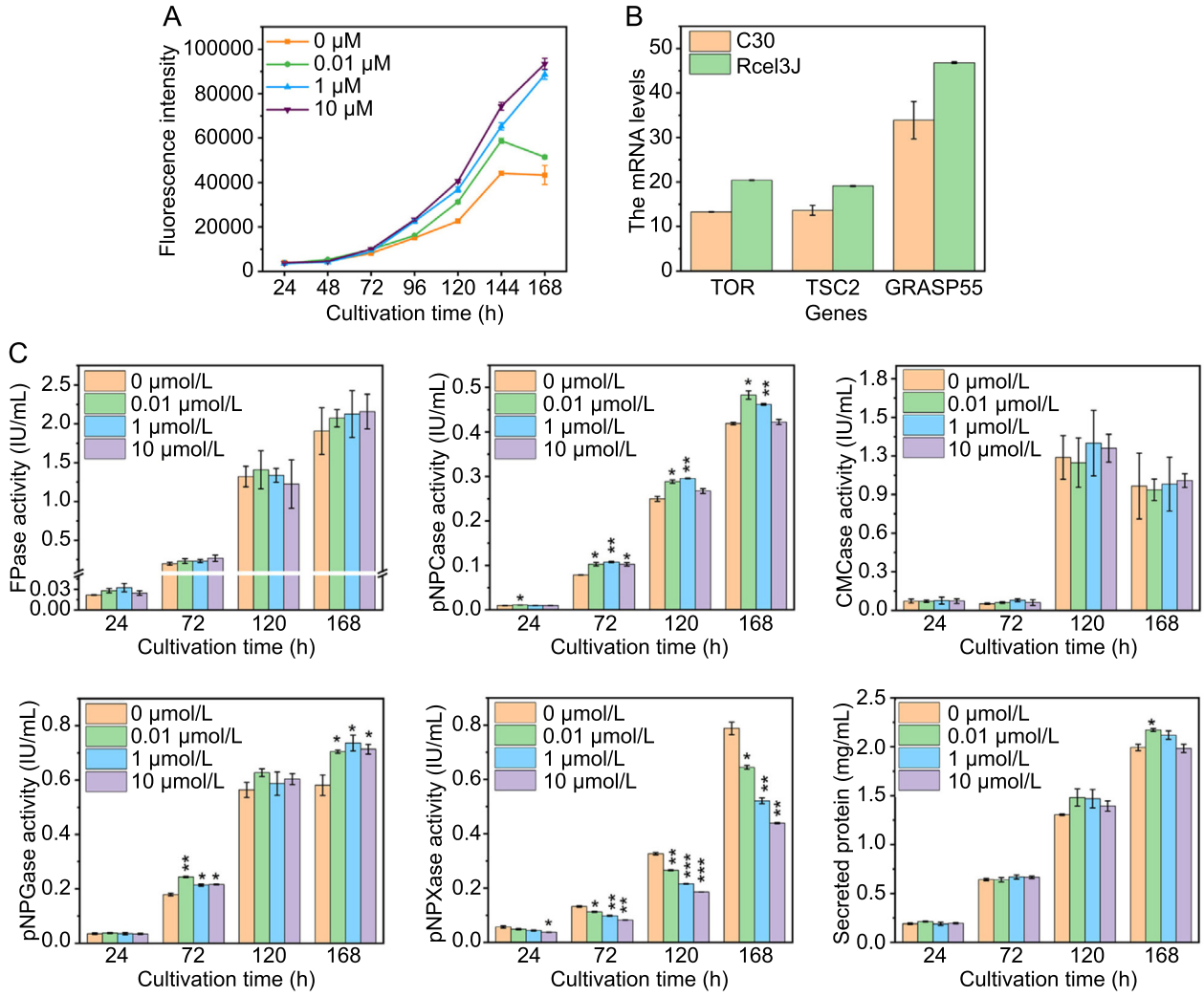


**Fig. 5.** (A) The impact of rapamycin on the fluorescence intensity of strain Rcel3J cultured on TMM + 2% cellulose. (B) The mRNA levels of the representative genes associated with mTORC1-GRASP55 in RUT-C30 and Rcel3J cultured on TMM + 2% cellulose for 120 h. (C) The cellulase activities and protein secretion of *T. reesei* Rcel3J cultured on TMM + 2% cellulose with the addition of different concentrations of rapamycin. Data are represented as the mean of three independent experiments, and error bars denote the standard deviations. Asterisks indicate significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) as assessed by the Student's *t*-test.

pression of none of the genes related to the UPS was decreased, except for *Atg8*. In particular, the expression of HSP70, TOR, TSC2, GRASP55, and TMED10 were notably increased with $p < 0.001$, indicating an active UPS in Rcel3J, which is consistent with the fact that both cellulase and CEL3J are secreted predominantly through the UPS in Rcel3J.

## 4. Discussion

In this study, we, for the first time, evaluated the effect of different β-glucosidases on cellulase activities and protein secretion in *T. reesei* using a single gene knockout. We found that the secretion of cellulase components (CBH, CMC, and β-glucosidases) was compromised to varying degrees in the deletion strains. The productions of CBH and β-glucosidases were significantly reduced in each △BGL grown on cellulose or lactose. In contrast, the production of CMC in the majority of △BGL was barely affected, with the exception of CMC, which was notably reduced in △cel3c and △cel3j strains cultivated on cellulose, and △cel1a, △cel3d, and △cel3j strains cultivated on lactose. This indicated that these β-glucosidases are more important for CBH and glucosidase production than for CMC. Moreover, the expression and secretion of cellulase components possess distinct regulatory mechanisms, as previously proposed [16,17]. In previous studies, deletion of the major extracellular β-glucosidase *cel3a* in *T. reesei* RL-P37 led to a lag in the extracellular protein level and CMC production on 1% cellulose, lactose, or cellobiose [8]. Deletion of intracellular β-glucosidase *cel1b* in *T. reesei* KU70 did not noticeably affect the cellulase activities on 1% lactose or cellobiose, but resulted in FPase and pNPCase activities being decreased by 48.6% and 30.0%, respectively, on 2% cellulose [15]. The double deletion of two β-glucosidases *cel3a* and *cel1b* also reduced cellulase production while using cellulose as the carbon source [13]. Distinct effects of β-glucosidase deletion on the three major cellulase components were observed. All the 11 β-glucosidases may contribute to cellulase synthesis in *T. reesei*.

Among the β-glucosidases tested, deletion of *cel3f* had the minimum inhibitory effect on cellulase production. However, endogenous overexpression of *cel3f* promoted pNPGase activity by 93% at 168 h in *T. reesei* cultivated on cellulose [16]. The substrate specific activities of CEL3F on pNPG, cellobiose, cellotriose, cellobiose, sophorose, and laminaribiose were lower than those of other β-glucosidases in *T. reesei* [65]. CEL3F was confirmed to be extracellular and distributed as separate secretory

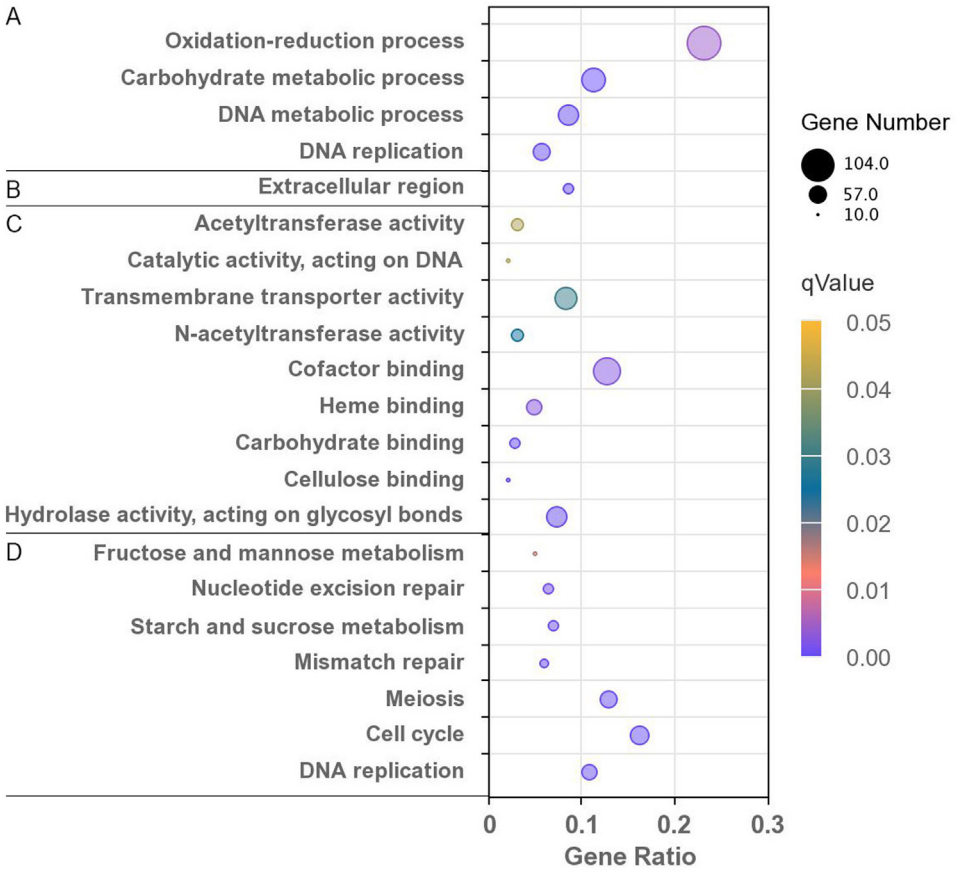


**Fig. 6.** Gene ontology (GO) functional enrichment (A-C) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment (D) analyses of differentially expressed genes (DEGs) in strain Rcel3J. (A) The most enriched biological processes; (B) The most enriched cellular components; (C) The most enriched molecular functions; (D) The most enriched KEGG pathways. The y-axis represents the name of the most enriched GOs or pathways that belong to different ontologies. Gene Ratio: the number of DEGs in a specified GO term/the number of the total DEGs in all GO terms; Gene Number: the number of DEGs in a specified GO term; q-value: *p* adjusted value.

vesicles in the cytoplasm, similar to CEL3A, CEL3B, CEL3E, and CEL3G [16]. In addition, the mRNA levels of *cel3f* were the lowest among all the *β*-glucosidases in *T. reesei* [16]. Thus, CEL3F may be functionally redundant, acting as an insurance mechanism for CEL3A to ensure maintenance of extracellular *β*-glucosidase activity in *T. reesei* for biomass degradation.

In contrast, the deletion of *cel3j* inhibited cellulase production the most severely when cultivated on both cellulose and lactose. This notable inhibitory effect resulting from *cel3j* deletion was also found on glucose, galactose, and glycerol, and showed no dependence on carbon sources, suggesting that *cel3j* has an essential role in cellulase production. Overexpression of *cel3j* did not affect pNPGase activity [16]. The mRNA levels of *cel3j* were not notably changed under cellulase-repressing conditions such as *T. reesei* RUT-C30 treated with 100 *μ*mol/L rapamycin [40] or 20 mmol/L glutamine [41], and in the *cel1b*-overexpressing *T. reesei* strain, Rcel1b [15], where the mRNA levels of most *β*-glucosidase genes were significantly changed. Thus, *cel3j* was required for effective cellulase production by *T. reesei*. Interestingly, in our study, deletion of *cel3g* reduced cellulase productivity, whereas it has been previously reported that knockout of *cel3g* increased cellulase productivity [6]. This discrepancy may be due to the varied experimental conditions of the parental strains and culture media. In a previous study, *T. reesei* uridine auxotrophic *ura5*-negative strain QmU2–3, screened from mutants of QM9414 (ATCC 26,921) by UV mutagenesis, was employed as the parental strain for *cel3g* deletion and the △*cel3g* strain were cultivated in minimal medium with 1% cellulose, different from RUT-C30 in TMM + 2% cellulose, as performed in this research.

Transcriptional analysis of △*cel3j* strain showed that the biological processes most affected were ribosomal biogenesis and RNA polymerase. The genes involved in ribosome biogenesis encode ribosomal components (Figure S6) and are associated with rRNA processing (Table S7). As ribosomes have long been known to be sites for nascent proteins that are crucial for cellulase synthesis, it is not surprising that genes involved in ribosome biogenesis were affected in △*cel3j* strain with significantly reduced cellulase production. Nevertheless, in contrast to decreased cellulase production, most genes related to ribosome biogenesis were significantly upregulated; however, the reason for this is unknown. Although the mRNA levels of genes involved in ribosome biogenesis increased, the mRNA levels of cellulase genes and DEGs related to ER (Figure S7) were notably reduced upstream and downstream of nascent protein synthesis in the ribosome, respectively. DEGs involved in ER-associated degradation were upregulated (Figure S7). These results may explain why cellulase synthesis was noticeably reduced in the *cel3j* strain. Genes associated with ribosome biogenesis in glutamine-treated *T. reesei* RUT-C30 with inhibited cellulase production were reduced compared to those in untreated RUT-C30 [41]. No direct positive correlation between the transcript levels of genes related to ribosome biogenesis and cellulase synthesis was observed.

CEL3J contains transmembrane helices and is located on the cell membrane [16]. The insensitivity of BFA to CEL3J secretion suggests that it is secreted via an unconventional protein secretion pathway [16]. In this study, we demonstrated that CEL3J is the only ER core that is glycosylated without Golgi-mediated complex *N*-glycosylation. Moreover, CEL3J was found in the ER, as shown by the colocalization of CEL3J-DsRed and ER-oriented GFP (Fig. 3). Overall, CEL3J was transported through the ER but not through the Golgi apparatus, demonstrating that the unconventional secretion of CEL3J was mediated by the Golgi bypass pathway. Interestingly, the unconventional secretion of CEL3J is associated with mTORC1-GRASP55. Rapamycin drastically induced CEL3J secretion in Rcel3J, and the transcription levels of TSC2, TOR, GRASP55, and HSP90 in Rcel3J showed obvious increases of 46.3%, 60.6%, 44.2%, and 109.5%, respectively, compared to that of those in RUT-C30. Based on this, we speculated that mTORC1-GRASP55 signaling pathway is

involved in CEL3J unconventional secretion. The activated mTORC1-GRASP55 pathway may further trigger the unconventional protein secretion of cellulase components that have been proposed to be capable of secreting unconventionally, leading to the resistance of cellulase secretion to BFA in strain Rcel3J (Fig. 5). As CEL3J is an unconventional secretion protein, its overexpression of CEL3J may activate the relevant unconventional protein secretion pathway, as previously observed [35]. This may explain why cellulase is predominantly secreted by an unconventional protein pathway in Rcel3J. Furthermore, this could also explain why cellulase production was improved at a low concentration of rapamycin (0.01 $\mu$mol/L) [40]. Low-dose rapamycin inhibited TOR and activated an unconventional secretion pathway, which could be exploited by cellulase for secretion, leading to improved cellulase production. It appears that the unconventional protein secretion pathway can be manipulated to modulate the secretion of cellulase, such as by the addition of rapamycin or overexpression of unconventional proteins such as CEL3J.

Two major characteristics were observed in Rcel3J. First, the secretion of cellulase and CEL3J occurred mainly via unconventional protein secretion, mediated by the mTORC1-GRASP55 signaling axis, which is different to the conventional protein secretion pathway of the parental strain RUT-C30. Second, the transcription levels of DEGs related to DNA replication, DNA repair (nucleotide excision repair and mismatch repair), the cell cycle, and meiosis were noticeably upregulated. Whether there is a relationship between the transcriptional upregulation of these DEGs and the switch in protein secretion mode is still unknown.

Notably, the study of $\beta$-glucosidases was performed using RUT-C30 as the parental strain, in which carbon catabolite repression was relieved to some extent owing to a truncated form of the *crel* gene (*crel-1*) [66,67]. Despite this, CCR was not completely abolished and still significantly expressed in RUT-C30 [12]. RUT-C30 is currently the basis of industrial cellulase production. Therefore, it is necessary to study the function of $\beta$-glucosidases in the production of cellulase by fungi using RUT-C30. However, for a better analysis of the role of $\beta$-glucosidases in cellulase induction, further experiments on strains QM6a or QM9414 are required.

In conclusion, we found that $\beta$-glucosidases facilitate (hemi)cellulase production with no observable influence on cell growth, sporulation, and mycelial morphology in *T. reesei*. In particular, deletion of *cel3j* severely inhibited cellulase production regardless of the carbon source, downregulating the genes known to be involved in cellulase synthesis, but upregulating genes for ribosome biogenesis and RNA polymerase activity. Unconventionally secreted CEL3J was shown to be transported through the ER, bypassing the Golgi apparatus, which is possibly regulated by the mTORC1-GRASP55 signaling axis. Moreover, the overexpression of *cel3j* altered the secretion of cellulase from conventional to unconventional and increased the mRNA levels of genes associated with DNA replication, DNA repair, the cell cycle, and meiosis. These findings deepen our knowledge of $\beta$-glucosidases and the unconventional secretion pathway in filamentous fungi.

## CRediT authorship contribution statement

APP, ZL, and FL conceived and designed the study. APP carried out the majority of the experiments. HW, YL and FZ carried out some experiments on *T. reesei* cultivation, enzyme activity, and confocal imaging respectively. FGW and ZZ helped analyze the data. APP and FL analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2023.100105.

## References

[1] S. Yan, Y. Xu, X.W. Yu, From induction to secretion: a complicated route for cellulase production in *Trichoderma reesei*, Bioresour. Bioprocess. 8 (2021) 107.
[2] K.S. Johansen, Lytic polysaccharide monooxygenases: the microbial power tool for lignocellulose degradation, Trends Plant Sci. 21 (2016) 9264–9936.
[3] A.C. dos Santos, E. Ximenes, Y. Kim, M.R. Ladisch, Lignin–enzyme interactions in the hydrolysis of lignocellulosic biomass, Trends Biotechnol. 37 (2019) 518–531.
[4] S. Aich, S. Datta, Engineering of a highly thermostable endoglucanase from the GH7 family of *Bipolaris sorokiniana* for higher catalytic efficiency, Appl. Microbiol. Biotechnol. 104 (2020) 3935–3945.
[5] F. Contreras, S. Pramanik, A.M. Rozhkova, I.N. Zorov, O. Korotkova, A.P. Sinitsyn, U. Schwaneberg, M.D. Davari, Engineering robust cellulases for tailored lignocellulosic degradation cocktails, Int. J. Mol. Sci. 21 (2020) 1589.
[6] G. Zou, Y. Jiang, R. Liu, Z. Zhu, Z. Zhou, The putative beta–glucosidase BGL3I regulates cellulase induction in *Trichoderma reesei*, Biotechnol. Biofuels 11 (2018) 314.
[7] J. Hoque, J. Haldar, Direct synthesis of dextran–based antibacterial hydrogels for extended release of biocides and eradication of topical biofilms, ACS Appl. Mater. Interfaces 9 (2017) 15975–15985.
[8] T. Fowler, R.D. Brown, Jr, The *bgl1* gene encoding extracellular beta–glucosidase from *Trichoderma reesei* is required for rapid induction of the cellulase complex, Mol. Microbiol. 6 (1992) 3225–3235.
[9] C. Li, F. Lin, L. Zhou, L. Qin, B. Li, Z. Zhou, M. Jin, Z. Chen, Cellulase hyper–production by *Trichoderma reesei* mutant SEU–7 on lactose, Biotechnol. Biofuels 10 (2017) 228.
[10] R.L. Mach, B. Seiboth, A. Myasnikov, R. Gonzalez, J. Strauss, A.M. Harkki, C.P. Kubicek, The *bgl1* gene of *Trichoderma reesei* QM 9414 encodes an extracellular, cellulose–inducible beta–glucosidase involved in cellulase induction by sophorose, Mol. Microbiol. 16 (1995) 687–697.
[11] J. Zhang, Y. Zhong, X. Zhao, T. Wang, Development of the cellulolytic fungus *Trichoderma reesei* strain with enhanced beta–glucosidase and filter paper activity using strong artificial cellobiohydrolase 1 promoter, Bioresour. Technol. 101 (2010) 9815–9818.
[12] C. Li, F. Lin, Y. Li, W. Wei, H. Wang, L. Qin, Z. Zhou, B. Li, F. Wu, Z. Chen, A beta–glucosidase hyper–production *Trichoderma reesei* mutant reveals a potential role of cel3D in cellulase production, Microb. Cell Fact. 15 (2016) 151.
[13] Q. Zhou, J. Xu, Y. Kou, X. Lv, X. Zhang, G. Zhao, W. Zhang, G. Chen, W. Liu, Differential involvement of beta–glucosidases from *Hypocrea jecorina* in rapid induction of cellulase genes by cellulose and cellobiose, Eukaryot. Cell 11 (2012) 1371–1381.
[14] J. Xu, G. Zhao, Y. Kou, W. Zhang, Q. Zhou, G. Chen, W. Liu, Intracellular beta–glucosidases CEL1a and CEL1b are essential for cellulase induction on lactose in *Trichoderma reesei*, Eukaryot. Cell 13 (2014) 1001–1013.
[15] A.–P. Pang, Y. Luo, X. Hu, F. Zhang, H. Wang, Y. Gao, S. Durrani, C. Li, X. Shi, F.–G.J.M.C.F. Wu, Transmembrane transport process and endoplasmic reticulum function facilitate the role of gene *cel1b* in cellulase production of *Trichoderma reesei*, Microb. Cell Fact. 21 (2022) 1–17.
[16] A.–P. Pang, H. Wang, Y. Luo, Z. Yang, Z. Liu, Z. Wang, B. Li, S. Yang, Z. Zhou, X. Lu, F.–G. Wu, Z. Lu, F. Lin, Dissecting Cellular Function and Distribution of beta–Glucosidases in *Trichoderma reesei*, MBio 12 (2021) e03671.
[17] C.C. Li, A.P. Pang, H. Yang, R.J. Lv, Z.H. Zhou, F.G. Wu, F.M. Lin, Tracking localization and secretion of cellulase spatiotemporally and directly in living *Trichoderma reesei*, Biotechnol. Biofuels 12 (2019) 200.
[18] J.E. Rothman, F.T. Wieland, Protein sorting by transport vesicles, Science 272 (1996) 227–234.
[19] E.A. Balmer, C. Faso, The road less traveled? Unconventional protein secretion at parasite–host interfaces, Front. Cell Dev. Biol. 9 (2021) 662711.
[20] C. Rabouille, V. Malhotra, W. Nickel, Diversity in unconventional protein secretion, J. Cell Sci. 125 (2012) 5251–5255.
[21] C. Rabouille, Pathways of unconventional protein secretion, Trends Cell Biol. 27 (2017) 230–240.

[22] J. Nüchel, M. Tauber, J.L. Nolte, M. Mörgelin, C. Türk, B. Eckes, C. Demetriades, M. Plomann, An mTORC1–GRASP55 signaling axis controls unconventional secretion to reshape the extracellular proteome upon stress, Mol. Cell 81 (2021) 3275–3293.
[23] A.G. Grieve, C. Rabouille, Golgi Bypass, Skirting around the heart of classical secretion, Cold Spring Harbor Perspect. Biol. 3 (2011) a005298.
[24] H.Y. Gee, J. Kim, M.G. Lee, Unconventional secretion of transmembrane proteins, Semin. Cell Dev. Biol. 83 (2018) 59–66.
[25] P. Stanley, Golgi glycosylation, Cold Spring Harb. Perspect. Biol. 3 (2011) a005199.
[26] J. Roth, Protein N–glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control, and cell interactions, Chem. Rev. 102 (2002) 285–303.
[27] J. Kim, H.Y. Gee, M.G. Lee, Unconventional protein secretion–new insights into the pathogenesis and therapeutic targets of human diseases, J. Cell Sci. 131 (2018) jcs213686.
[28] G. Giuliani, F. Giuliani, T. Volk, C. Rabouille, The Drosophila RNA–binding protein HOW controls the stability of dgrasp mRNA in the follicular epithelium, Nucleic. Acids. Res. 42 (2014) 1970–1986.
[29] H. Schotman, L. Karhinen, C. Rabouille, dGRASP–mediated noncanonical integrin secretion is required for drosophila epithelial remodeling, Dev. Cell 14 (2008) 171–182.
[30] Z.–H. Wang, C. Rabouille, E.R. Geisbrecht, Loss of a Clueless–dGRASP complex results in ER stress and blocks Integrin exit from the perinuclear endoplasmic reticulum in Drosophila larval muscle, Biol. Open 4 (2015) 636–648.
[31] T.A. Baldwin, H.L. Ostergaard, The protein–tyrosine phosphatase CD45 reaches the cell surface via Golgi–dependent and –independent pathways, J. Biol. Chem. 277 (2002) 50333–50340.
[32] H. Hoffmeister, K. Babinger, S. Gürster, A. Cedzich, C. Meese, K. Schadendorf, L. Osten, U. de Vries, A. Rascle, R. Witzgall, Polycystin–2 takes different routes to the somatic and ciliary plasma membrane, J. Cell Biol. 192 (2011) 631–645.
[33] C. Cleyrat, A. Darehshouri, M.P. Steinkamp, M. Vilaine, D. Boassa, M.H. Ellisman, S. Hermouet, B.S. Wilson, Mpl traffics to the cell surface through conventional and unconventional routes, Traffic 15 (2014) 961–982.
[34] H. Park, D.H. Shin, J.R. Sim, S. Aum, M.G. Lee, IRE1 alpha kinase–mediated unconventional protein secretion rescues misfolded CFTR and pendrin, Sci. Adv. 6 (2020) eaax9914.
[35] Heon Y. Gee, Shin H. Noh, Bor L. Tang, Kyung H. Kim, Min G. Lee, Rescue of ΔF508–CFTR trafficking via a GRASP–dependent unconventional secretion pathway, CellCell 146 (2011) 746–760.
[36] M. Zhang, S.J. Kenny, L. Ge, K. Xu, R. Schekman, Translocation of interleukin–1 beta into a vesicle intermediate in autophagy–mediated secretion, Elife 4 (2015) 11205.
[37] J. Jung, J. Kim, S.H. Roh, I. Jun, R.D. Sampson, H.Y. Gee, J.Y. Choi, M.G. Lee, The HSP70 co–chaperone DNAJC14 targets misfolded pendrin for unconventional protein secretion, Nat. Commun. 7 (2016) 11386.
[38] M. Zhang, L. Liu, X. Lin, Y. Wang, Y. Li, Q. Guo, S. Li, Y. Sun, X. Tao, D. Zhang, X. Lv, L. Zheng, L. Ge, A translocation pathway for vesicle–mediated unconventional protein secretion, CellCell 181 (2020) 637–652.
[39] C. Wu, R. Zhang, W. Du, L. Cheng, G. Liang, Alkaline phosphatase–triggered self–assembly of near–infrared nanoparticles for the enhanced photoacoustic imaging of tumors, Nano Lett. 18 (2018) 7749–7754.
[40] A.-P. Pang, H.Y. Wang, F.N. Zhang, X. Hu, F.G. Wu, Z.H. Zhou, W. Wang, Z.H. Lu, F.M. Lin, High–dose rapamycin exerts a temporary impact on *T. reesei* RUT–C30 through gene *trFKBP12*, Biotechnol. Biofuels 14 (2021) 77.
[41] A.P. Pang, F.N. Zhang, X. Hu, Y.S. Luo, H.Y. Wang, S. Durrani, F.G. Wu, B.Z. Li, Z.H. Zhou, Z.H. Lu, F.M. Lin, Glutamine involvement in nitrogen regulation of cellulase production in fungi, Biotechnol. Biofuels 14 (2021) 199.
[42] Y.H. Zhong, X.L. Wang, T.H. Wang, Q. Jiang, *Agrobacterium*–mediated transformation (AMT) of *Trichoderma reesei* as an efficient tool for random insertional mutagenesis, Appl. Microbiol. Biotechnol. 73 (2007) 1348–1354.
[43] C. Wu, Y.M. Chen, X.X. Huang, S.S. Sun, J.N. Luo, Z.W. Lu, W. Wang, Y.S. Ma, An efficient shortened genetic transformation strategy for filamentous fungus *Trichoderma reesei*, J. Gen. Appl. Microbiol. 65 (2019) 301–307.
[44] C. Derntl, R.L. Mach, A.R. Mach–Aigner, Fusion transcription factors for strong, constitutive expression of cellulases and xylanases in *Trichoderma reesei*, Biotechnol. Biofuels 12 (2019) 231.
[45] M. Saloheimo, M. Paloheimo, S. Hakola, J. Pere, B. Swanson, E. Nyyssonen, A. Bhatia, M. Ward, M. Penttila, Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials, Eur. J. Biochem. 269 (2002) 4202–4211.
[46] R.C. Kuhad, D. Deswal, S. Sharma, A. Bhattacharya, K.K. Jain, A. Kaur, B.I. Pletschke, A. Singh, M. Karp, Revisiting cellulase production and redefining current strategies based on major challenges, Renew. Sustain. Energy Rev. 55 (2016) 249–272.
[47] T.M. Mello–de–Sousa, A. Rassinger, C. Derntl, M.J. Pocas–Fonseca, R.L. Mach, A.R. Mach–Aigner, The relation between promoter chromatin status, Xyr1 and cellulase expression in *Trichoderma reesei*, Curr. Genomics 17 (2016) 145–152.
[48] A.C.C. Antonieto, K.M.V. Nogueira, R.G.d. Paula, L.C. Nora, M.H.A. Cassiano, M.–E. Guazzaroni, F. Almeida, T.A.d. Silva, L.N.A. Ries, L.J.d. Assis, G.H. Goldman, R.N. Silva, R. Silva–Rocha, C. Vickers, A Novel Cys2His2 Zinc Finger Homolog of AZF1 Modulates Holocellulase Expression in *Trichoderma reesei*, 4 (2019) e00161.
[49] C. Ivanova, J. Ramoni, T. Aouam, A. Frischmann, B. Seiboth, S.E. Baker, S.Le Crom, S. Lemoine, A. Margeot, F. Bidard, Genome sequencing and transcriptome analysis of *Trichoderma reesei* QM9978 strain reveals a distal chromosome translocation to be responsible for loss of *vib1* expression and loss of cellulase induction, Biotechnol. Biofuels 10 (2017) 209.
[50] F. Zhang, X. Zhao, F. Bai, Improvement of cellulase production in *Trichoderma reesei* Rut–C30 by overexpression of a novel regulatory gene *Trvib–1*, Bioresour. Technol. 247 (2018) 676–683.
[51] M. Wang, Y. Dong, Q. Zhao, F. Wang, K. Liu, B. Jiang, X. Fang, Identification of the role of a MAP kinase Tmk2 in *Hypocrea jecorina* (*Trichoderma reesei*), Sci. Rep. 4 (2014) 6732.
[52] W. Zhang, Y. Kou, J. Xu, Y. Cao, G. Zhao, J. Shao, H. Wang, Z. Wang, X. Bao, G. Chen, W. Liu, Two major facilitator superfamily sugar transporters from *trichoderma reesei* and their roles in induction of cellulase biosynthesis, J. Biol. Chem. 288 (2013) 32861–32872.
[53] V. Novy, F. Nielsen, B. Seiboth, B. Nidetzky, The influence of feedstock characteristics on enzyme production in *Trichoderma reesei*: a review on productivity, gene regulation and secretion profiles, Biotechnol. Biofuels 12 (2019) 238.
[54] L. Ma, L. Chen, L. Zhang, G. Zou, R. Liu, Y. Jiang, Z. Zhou, RNA sequencing reveals Xyr1 as a transcription factor regulating gene expression beyond carbohydrate metabolism, Biomed. Res. Int. (2016) 4841756.
[55] R. Karimi Aghcheh, Z. Nemeth, L. Atanasova, E. Fekete, M. Paholcsek, E. Sándor, B. Aquino, I.S. Druzhinina, L. Karaffa, C.P.J.P.o. Kubicek, The VELVET A orthologue VEL1 of *Trichoderma reesei* regulates fungal development and is essential for cellulase gene expression, PLoS ONE 9 (2014) e112799.
[56] Q.–S. Meng, F. Zhang, C.–G. Liu, X.–Q. Zhao, F.–W. Bai, Identification of a novel repressor encoded by the putative gene ctf1 for cellulase biosynthesis in *Trichoderma reesei* through artificial zinc finger engineering, Biotechnol. Bioeng. 117 (2020) 1747–1760.
[57] H.R.B. Pelham, The retention signal for soluble-proteins of the endoplasmic-reticulum, Trends Biochem. Sci 15 (1990) 483–486.
[58] M. Terasaki, T.S. Reese, Characterization of endoplasmic-reticulum by colocalization of Bip and dicarbocyanine dyes, J. Cell Sci. 101 (1992) 315–322.
[59] A. González, M.N. Hall, S.C. Lin, D.G. Hardie, AMPK and TOR: the Yin and Yang of cellular nutrient sensing and growth control, Cell Metab. 31 (2020) 472–492.
[60] A. Bakshi, M. Moin, M.S. Madhav, P.B. Kirti, Target of rapamycin, a master regulator of multiple signalling pathways and a potential candidate gene for crop improvement, Plant Biol. (Stuttgart, Germany) 21 (2019) 190–205.
[61] J. Kim, K.L. Guan, mTOR as a central hub of nutrient signalling and cell growth, Nat. Cell Biol. 21 (2019) 63–71.
[62] D. Cruz–Garcia, V. Malhotra, A.J. Curwin, Unconventional protein secretion triggered by nutrient starvation, Semin. Cell Dev. Biol. 83 (2018) 22–28.
[63] A.J. Curwin, N. Brouwers, M. Alonso Y Adell, D. Teis, G. Turacchio, S. Parashuraman, P. Ronchi, V. Malhotra, ESCRT–III drives the final stages of CUPS maturation for unconventional protein secretion, Elife 5 (2016) e16299.
[64] M.C. Giraldo, Y.F. Dagdas, Y.K. Gupta, T.A. Mentlak, M. Yi, A.L. Martinez–Rocha, H. Saitoh, R. Terauchi, N.J. Talbot, B. Valent, Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*, Nat. Commun. 4 (2013) 1996.
[65] B. Guo, N. Sato, P. Biely, Y. Amano, K. Nozaki, Comparison of catalytic properties of multiple beta–glucosidases of *Trichoderma reesei*, Appl. Microbiol. Biotechnol. 100 (2016) 4959–4968.
[66] T.M. Mello-de-Sousa, R. Gorsche, A. Rassinger, M.J. Poças-Fonseca, R.L. Mach, A.R. Mach-Aigner, A truncated form of the Carbon catabolite repressor 1 increases cellulase production in *Trichoderma reesei*, Biotechnol. Biofuels 7 (1) (2014) 129.
[67] M. Ilmén, C. Thrane, M. Penttilä, The glucose repressor gene *cre1* of Trichoderma: isolation and expression of a full-length and a truncated mutant form, Mol. Gen. Genet. 251 (4) (1996) 451–460.