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Improved Cellulase Production of *Trichoderma reesei* by Regulating Mycelium Morphology

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Abstract: The small GTPases of the Rho family are known to regulate various biological processes in filamentous fungi. In this study, we investigated the impact of deleting Rho proteins on the growth and cellulase production of *Trichoderma reesei*. Our findings revealed that deletion of *cdc42* led to the most severe growth defect and impaired cellulase production. Conversely, overexpression of *cdc42* resulted in a hyperbranched phenotype, significantly enhancing cellulase production. Furthermore, the *cdc42*-overexpressing (OCdc42) strain showed an increased expression of multiple cellulase genes and Rho GTPase genes. Analysis of the secretome in the OCdc42 strain unveiled an increased abundance and diversity of extracellular proteins compared to the parent strain. These discoveries provide valuable insights into the functionality of Rho GTPases in *T. reesei* and offer potential targets for engineering fungi to improve plant biomass deconstruction in biorefineries.

Keywords: *Trichoderma reesei*; small GTPase; Cdc42; hyperbranched phenotype; cellulase

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1. Introduction

Filamentous fungi are highly efficient microorganisms for industrial protein production due to their remarkable ability to express and secrete large amounts of protein [1]. They are widely utilized in the production of enzyme products, which play a crucial role in the food processing and bioenergy industries. Among these fungi, *Trichoderma reesei* stands out as a leading producer of cellulase. Protein secretion in filamentous fungi is closely linked to hyphal tip growth [2]. The rapid growth of mycelium requires constant synthesis of cell walls and the transport of cell membrane components, organelles, and vesicles. Establishing and maintaining cellular polar growth is crucial to facilitate the apical secretion of extracellular protein [3]. Extensive research has demonstrated that hyperbranching is associated with an enhanced capacity for protein secretion by increasing the number of tips [4,5]. Additionally, the hyperbranched phenotype leads to lower viscosity during liquid/solid fermentation, thereby improving the availability of dissolved oxygen and subsequently increasing protein yield [6,7].

The Ras homologue (Rho) proteins, which belong to the Ras GTPase superfamily, play crucial roles in various biological processes, including metabolism, morphogenesis, cell polarity, cytoskeletal organization, and gene expression. Rho GTPases are highly conserved in all eukaryotes, with a total of 20 members identified [8]. Among these members, RhoA, RhoB, RhoC, RhoD, RacA, and Cdc42 are typically the most dominant in filamentous fungi [9]. RhoA has been extensively studied as a key regulator of polarity establishment and cell viability [9–14]. RhoB is involved in various processes in different fungal species.

RhoB is implicated in sporulation and cell wall integrity in *Aspergillus niger* [9]. In *Colletotrichum gloeosporioides*, RhoB is involved in conidial germination, septum formation, cell wall integrity, and chitin distribution [15]. In *Ashbya gossypii*, RhoB is associated with tip-branching [16]. In *Magnaporthe oryzae*, RhoB is involved in the morphological development of appressoria and virulence [17]. RhoB also contributes to cell wall integrity in *Neurospora crassa*, *Arthrobotrys oligospora*, and *Fusarium graminearum* [13,14,18]. While RhoC had no obvious function in *A. niger* [9], its homolog is involved in growth, conidiation, and virulence in *Botrytis cinerea* [19], secretion process in *T. reesei* [20], appressorium formation and polar growth in *M. oryzae* [21], and vegetative growth and conidiation in *F. graminearum* [14], as well as cell polarity and hyphal morphogenesis in *A. gossypii* [10]. RhoD is required for sporulation, septum formation, and cell wall integrity in *A. niger* and *A. nidulans* [9,22], hyphal growth, conidiation, and septum formation in *F. graminearum* [14], septum formation, hyphal tip growth, and possibly cell wall integrity in *N. crassa* [23,24], and cell wall integrity and virulence in *Colletotrichum graminicola* [25].

RacA and Cdc42 collectively maintain cell polarity, as simultaneous deletion of RacA and Cdc42 is lethal in *A. niger*, *Ustilago maydis*, and *N. crassa* [9,26,27]. RacA and Cdc42 display both overlapping and independent functions. Deletion of *racA* in *A. niger*, *Colletotrichum scovillei*, *Claviceps purpurea*, *Aspergillus fumigatus*, *Nomuraea rileyi*, *Magnaporthe grisea*, *F. graminearum*, *A. oligospora*, *Epichloë festucae*, *N. crassa*, *Aspergillus flavus*, and *T. reesei* results in increased hyphal branching, growth defect, and/or loss of cell polarity [9,18,26,28–38]. However, the loss of Cdc42 does not significantly affect hyphal morphogenesis in *A. niger* [9]. In contrast, Cdc42 plays a major role in hyphal morphogenesis and establishment of hyphal polarity, while Rac1 is largely dispensable in *A. nidulans* [39,40]. Cdc42 is involved in diverse biological processes in filamentous fungi, such as polarity establishment in *Penicillium marneffei* [41], *A. gossypii* [10], *Schizophyllum commune* [42], and *N. crassa* [26], and growth or morphological development in *U. maydis* [27], *Colletotrichum trifolii* [43], *C. purpurea* [44], *C. gloeosporioides* [45], *E. festucae* [36], *N. crassa* [46], *A. oligospora* [18], *F. graminearum* [14], and *B. cinerea* [47], as well as cytokinesis [27], virulence [31,34,43,44,47], conidiation [14,34], germination [43,47], reactive oxygen species (ROS) production [18,31,32,34,43,45], and pyruvate metabolism [48]. However, the specific effects of Cdc42 on morphology and protein production in *T. reesei* have not been investigated.

In this study, the function of Cdc42 and other small GTPase members was investigated in *T. reesei*. In particular, the effect of *cdc42* overexpression on fungal growth, hyphal branching, cellulase gene expression, and protein production was investigated. Our findings provide new insights into the function of Ras GTPase in cellulolytic filamentous fungi and provide potential targets for optimizing fungal strains for more efficient and cost-effective biomass conversion in biorefineries.

2. Materials and Methods

2.1. Fungal Strains, Media, and Cultivation Conditions

Trichoderma reesei strain A2H, which exhibits a high cellulase production phenotype, was obtained by ARTP (atmospheric and room temperature plasma) mutagenesis in our laboratory [49]. This strain was preserved at the China General Microbiological Culture Collection Center (CGMCC 21470) and was used as a parent strain in this study. *T. reesei* strains were cultured at 30 °C for 7 days to obtain conidia. The growth medium consisted of 2% glucose, 1.5% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.06% $CaCl_2$, and 0.06% $MgSO_4$. For the inoculum medium, 2% Avicel, 0.2% glucose, and 1.7% corn steep liquor were used. Medium pH was adjusted to 4.5 with 2 M KOH. For fermentation, the conidia of *T. reesei* were inoculated into 100 mL of medium consisting of 3.3% Avicel, 1.7% corn steep liquor, 0.6% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.1% $MgSO_4$, 0.25% glycerol, 0.25% $CaCO_3$, and 0.1% Tween-80 (pH 5.0, adjusted with 2 M KOH) in a 250-mL Erlenmeyer flask. Selection medium (30 g/L malt extract, 182 g/L sorbitol, 20 g/L agarose) was used for screening transformants after protoplast transformation.

2.2. Plasmid Construction

The primers used in this study are listed in Table S1. For gene deletion, the 1-kb regions upstream of the start codon of *cdc42* (TRIREDRAFT_50335), *cla4* (TRIREDRAFT_71315), *ras1* (TRIREDRAFT_120150), *ras2* (TRIREDRAFT_110960), *spa2* (TRIREDRAFT_108829), *rho1* (TRIREDRAFT_119871), and *rac1* (TRIREDRAFT_47055) were amplified from the genome of *T. reesei* using primers cdc42-up-F/R, cla4-up-F/R, ras1-up-F/R, ras2-up-F/R, spa2-up-F/R, rho1-up-F/R, and rac1-up-F/R, respectively. And the 1-kb regions downstream of the corresponding genes were amplified using primers cdc42-down-F/R, cla4-down-F/R, ras1-down-F/R, ras2-down-F/R, spa2-down-F/R, rho1-down-F/R, and rac1-down-F/R, respectively. The hygromycin B-resistance gene (*hph*) was amplified from plasmid pCamhy-bgfp1 (GenBank accession no. KX223837) using primers hph-F/R. The upstream (5′) and downstream (3′) region of each gene and *hph* fragment were assembled into the pEASY-Blunt Simple cloning vector (TransGen Biotech, Beijing, China) using the ClonExpress Ultra One Step Cloning Kit (Vazyme Biotech, Nanjing, China). The 5′-*hph*-3′ cassette was amplified using primers M13F/M13R and used for protoplast transformation of the A2H strain.

For overexpression of *cdc42*, the promoter region of *tef1a* (TRIREDRAFT_46958) (P*tef1*) and the terminator region of *egl1* (*cel7b*, TRIREDRAFT_122081) (T*egl1*) were amplified from *T. reesei* genomic DNA with the primers Ptef-F/Ptef-R and Tegl1-F/R, respectively. The open reading frame (ORF) of *cdc42* was amplified from *T. reesei* cDNA using primers cdc42-F/cdc42-R. The 2A peptide-coding sequence and the enhanced green fluorescent protein (eGFP) gene were amplified from the plasmid P*tef1*-MhGlaA-9 × His-2A-GFP-T*trpC* [50] using primers 2A-F/eGFP-R. These fragments were then assembled into the pEASY-Blunt Simple cloning vector. The P*tef1-cdc42*-2A-eGFP-T*egl1* cassette was amplified with primers Ptef-F/Tegl1-R and used for protoplast transformation of the A2H strain.

2.3. PEG-Mediated Transformation of T. reesei

Conidia of *T. reesei* were harvested and inoculated into 100 mL of growth medium. After incubation at 28 °C with shaking (180 rpm) for 18 h, mycelia were harvested and used for preparing the protoplasts. Protoplasts of *T. reesei* were prepared as previously described [51]. The transformed protoplasts were either grown for 3–4 days on selection medium at 28 °C, with selection for *hph* resistance using hygromycin B (50 μg/mL), or they were screened by flow cytometry analysis.

2.4. Screening of Transformants by Flow Cytometry Analysis

The transformed protoplasts were grown overnight in 20 mL liquid growth medium supplemented with 1 M sorbitol. The freshly regenerated hyphae were harvested, rinsed, and suspended in 3 mL of 0.05% (*w*/*v*) Tween 80. After filtration through a 70 μm Cell Strainer (Wuxi NEST Biotechnology Co., Ltd., Wuxi, China), hyphae were sorted using a MoFlo™ XDP cell sorter (Beckman Coulter Inc., Brea, CA, USA). Fluorescence-based screening was performed according to the manufacturer's instructions. Single hyphae with the brightest GFP signal were sorted into individual wells (each well contained 150 μL of growth medium) of 96-deep-well plates and incubated at 28 °C with shaking (130 rpm) for 48 h. The transformants were then reexamined to pick up those with the brightest GFP signal.

2.5. Growth Test

Approximate 1×10^6 spores of strains were inoculated on PDA and minimal medium supplemented with soluble starch, sucrose, glucose, and Avicel. Photos were taken after culture at 30 °C for 5 days.

2.6. Protein and Enzyme Assays

Extracellular proteins were determined by SDS-PAGE analysis. Protein concentrations were estimated using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham,

MA, USA). FPase activity was measured as previously described [52]. The β-glucanase activity of the culture supernatants was detected as previously described [53] using 1.0% carboxymethylcellulose sodium (CMC-Na) as the substrate. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

2.7. RNA Isolation and Quantitative Real-Time PCR

Fungal mycelia were harvested, washed, and frozen. They were then ground under liquid nitrogen. Total RNA was isolated using an RNAprep Pure Plant Kit (TIANGEN Biotech Co., Ltd., Beijing, China). The removal of genomic DNA was confirmed by reverse transcription-PCR (RT-PCR). cDNA synthesis was performed using a TransScript® Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech Co., Ltd., Beijing, China). Quantitative real-time PCR (qPCR) assays were performed as previously described [54]. Primers used for qRT-PCR are listed in Table S1. Samples were analyzed in three independent experiments. The relative expression levels of the target genes were standardized against the levels of β-actin gene using the $2^{-\Delta\Delta CT}$ method [55].

3. Results and Discussion

3.1. Deletion of cdc42 Leads to Growth Defect and Impaired Cellulase Production

Ras homology (RHO) GTPases are signaling proteins that play a critical role in various biological functions, such as cell polar growth and cell morphogenesis. In filamentous fungi, the activity of these enzymes is particularly important in controlling hyphal morphogenesis, a crucial aspect of fungal development. The hyperproducing cellulase mutant *T. reesei* A2H was obtained from the parent strain RUT C30 using diethyl sulfite (DES) mutagenesis in our laboratory [49]. The expression of seven genes that have previously been reported to be involved in the branching process (*cla4*, *rho1*, *spa2*, *rac1*, *ras1*, *ras2*, and *cdc42*) was analyzed. Previous work suggested that the downregulation of these genes may contribute to the highly branched hyphae in the DES-15 strain [54].

The expression levels of *cla4*, *ras1*, *ras2*, *spa2*, *rho1*, *cdc42*, and *rac1* were significantly decreased in the A2H strain compared to the parental RUT C30 strain. To investigate the role of these genes in mycelial growth, each of these genes was deleted in the *T. reesei* A2H strain. Compared to the parental A2H strain, all deletion strains exhibited smaller colony sizes on the PDA plate (Figure 1A). These findings provide additional support for the essential role of these genes in hyphal growth in ascomycetous fungi. Interestingly, the Δ*cdc42* mutant exhibited a significant reduction in elongation rate and formed smaller but thicker colonies compared to the other mutants (Figure 1A). In addition, the Δ*cdc42* mutant showed the most significant decrease in cellulase activity (Figure 1B). This suggests that Cdc42 may be involved not only in the regulation of mycelial morphology but also in cellulase production. Cdc42 in *Saccharomyces cerevisiae* is essential for regulating and organizing the actin cytoskeleton, which is required for directed plasma membrane and maintenance of cell polarity [56]. Cdc42 in filamentous fungi played a similar function during isotropic spreading and was restricted to the bud during the polar extension. However, mutations in genes that maintain polarity often lead to a split mycelial tip in filamentous fungi, a phenotype that has no analog in yeast. Among them, *cdc42* is a key gene involved in the establishment and maintenance of cell polarity. In this study, the knockout of *cdc42* resulted in a significant reduction in hyphal elongation, smaller colony size, and slower hyphal growth. The delay and variation in spore polarization could be attributed to the crucial role of *cdc42* in establishing polarity, while other genes such as *cla4*, *ras2*, *ras1*, *spa2*, *rho1*, and *rac1* are involved in maintaining polarity.

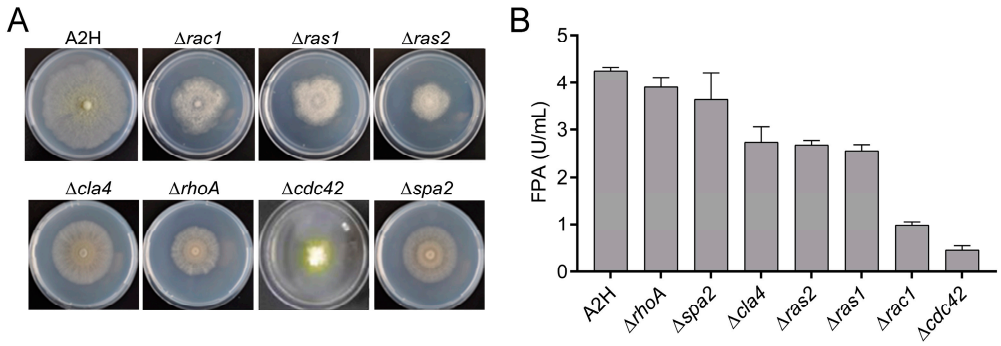

Figure 1. Characterization of gene deletion mutants for *cla4*, *ras2*, *ras1*, *spa2*, *rho1*, *cdc42*, and *rac1*. (**A**) Growth of the parent strain A2H and gene deletion mutants on PDA medium for 5 days at 30 °C. (**B**) Filter paper activity of the parent strain A2H and gene deletion mutants.

3.2. Cdc42 Function in Polarized Apical Growth and Branching

The hyphae of the Δ*cdc42* mutant also appeared to be more linear and less branched than those of the parent strain (Figure 1A). To further investigate the role of Cdc42, we constructed a *cdc42* overexpression cassette and introduced it into the *T. reesei* A2H strain. To conveniently screen out the positive transformants, we designed a co-expression system of Cdc42 and eGFP using the 2A peptide [57]. The 2A peptide is a self-cleaving peptide that enables the simultaneous production of eGFP and Cdc42. The transformed protoplasts with the highest GFP fluorescence were selected using a flow cytometer (Figure S1). One of the transformants with the highest GFP signal after re-assessment was selected as a *cdc42*-overexpressing strain (OCdc42). The OCdc42 strain grew better than the A2H strain on PDA medium and on Avicel (Figure 2A). After 72 h of cultivation in a glucose-containing medium, the hyphal branching of the parent strain and the OCdc42 strain were recorded. The results showed that the OCdc42 strain exhibited a higher frequency of hyphal branching (23–56 μm/tips) compared to the parent strain (73–114 μm/tips) (Figure 2B,C), leading to an increased number of mycelial tips which may in turn promote enzyme production.

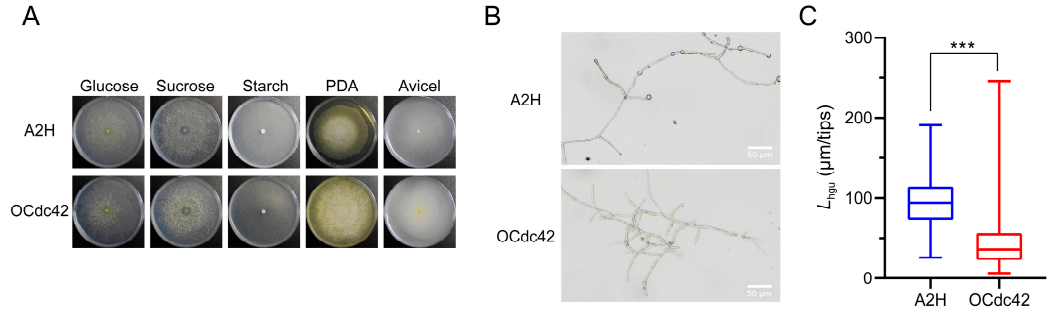

Figure 2. Overexpression of *cdc42* results in hyper branching. (**A**) Growth of A2H strain and OCdc42 strain on different medium. (**B**) Mycelial branching of A2H strain and OCdc42 strain. (**C**) The hyphal growth unit (L_{hgu}, the ratio of total mycelial length to total number of hyphal tips) of the A2H strain (blue) and OCdc42 strain (red). *** $p < 0.001$.

3.3. Enhanced Protein Secretion and Cellulase Genes Expression in OCdc42 Strain

Cellulase production is one of the most important cellular processes for *T. reesei*, as this fungus is widely known for its exceptional capacity to produce significant amounts of cellulases [58]. In this regard, the OCdc42 strain shows a significant increase in the secretion of extracellular proteins by 50.2% (13.7 ± 0.4 g/L vs. 9.1 ± 0.7 g/L, $p < 0.001$) (Figure 3A,B), along with a 20.9% increase in FPAase activity (5.2 ± 0.3 U/mL vs. 4.3 ± 0.1 U/mL, $p < 0.01$) (Figure 3C), and a 39.1% increase in endoglucanase activity (46.1 ± 2.0 U/mL vs. 33.1± 1.7 U/mL, $p < 0.001$) (Figure 3D), compared to the control. These observations strongly suggest that Cdc42 plays an important role in the production of cellulase.

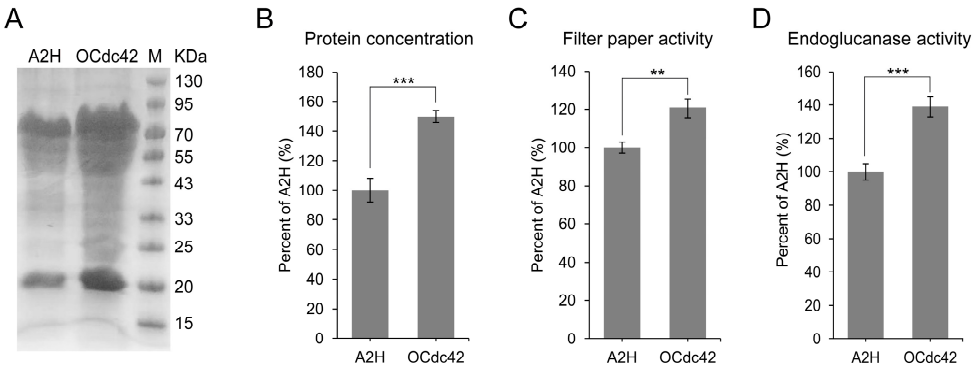

Figure 3. Overexpression of *cdc42* results in elevated protein secretion. (**A**) SDS-PAGE of proteins secreted by the A2H strain and OCdc42 strain. (**B**) Extracellular protein concentration in culture supernatants of A2H and OCdc42 strains after 4 days of growth in 2% (*w*/*v*) Avicel medium. (**C**) Filter paper activity (FPA) in culture supernatants of A2H and OCdc42 strains. (**D**) Endoglucanase activity in culture supernatants of A2H and OCdc42 strains. ** $p < 0.01$; *** $p < 0.001$.

Multiple transcription factors are responsible for the regulation of cellulase and xylanase genes [58]. In cellulase-hyperproducing strains of *T. reesei*, the expression of transcription factors and genes involved in cellulase production is usually upregulated, resulting in high cellulase production [59]. For example, the transcript abundance of *xyr1* and *cbh1* was significantly higher in the industrial cellulase-hyperproducing strain *T. reesei* CL847 than in its parent strain RUT C30 [60]. Consistent with the elevated cellulase production (Figure 3), the expression levels of multiple cellulase genes, including *cel7b* (TRIREDRAFT_122081), *cel3a* (TRIREDRAFT_76672), *cel3d* (TRIREDRAFT_46816), *cel3c* (TRIREDRAFT_82227), *cel1a* (TRIREDRAFT_120749), *cel45a* (TRIREDRAFT_49976), *cel12a* (TRIREDRAFT_123232), *cel61a* (TRIREDRAFT_73643), *gh31-1* (TRIREDRAFT_82235), and *bxl1* (TRIREDRAFT_121127) in the OCdc42 strain, are significantly higher than that in the A2H strain (Figure 4).

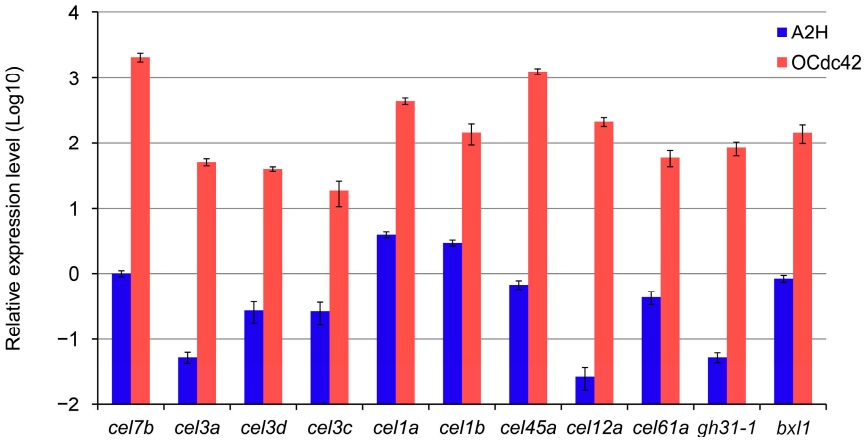

Figure 4. Expression levels of various cellulase genes in A2H and OCdc42 strains.

Meanwhile, the proliferation of hyphal branches stimulates the synthesis of secreted proteins. Consequently, a notable increase in extracellular protein levels was observed in the *cdc42*-overexpressing strain (OCdc42). Notably, the OCdc42 strain exhibited a faster growth rate than its parent strain. Furthermore, the OCdc42 strain generated larger and more transparent circles on cellulose medium, indicating a notable advantage in enzyme production. In addition, the major cellulase genes were significantly upregulated in the OCdc42 strain, indicating its involvement in the regulation of cellulase gene expression. The identification of the downstream components of the Cdc42 pathway in cellulase gene expression will be a critical step in understanding the regulatory mechanisms involved in cellulase production.

3.4. Exoproteome Analysis of Secreted Proteins

SDS-PAGE analysis revealed a significant retention of certain bands in the OCdc42 strain compared to the A2H strain (Figure 3A). To investigate the differences in the secretomes of the OCdc42 and A2H strains, we further delineated the proteome by identification of extracellular proteins in the supernatants of 3-day Avicel cultures through LC-MS/MS analysis. A total of 54 proteins were detected in the OCdc42 strain, whereas only 30 proteins were identified in the A2H strain (Table S2). In the secretome analyses, 29 proteins were common to both strains (Figure 5A), of which 24 proteins showed increased abundance in the OCdc42 strain (Figure 5B, Table S2). Notably, 25 proteins were exclusively secreted by the OCdc42 strain, including 1 cell wall protein, 23 CAZymes, and 1 superoxide dismutase. The OCdc42 strain exhibits enhanced protein secretion, which aligns with its superior growth on Avicel medium (Figure 2A) and increased enzymatic activity (Figure 3).

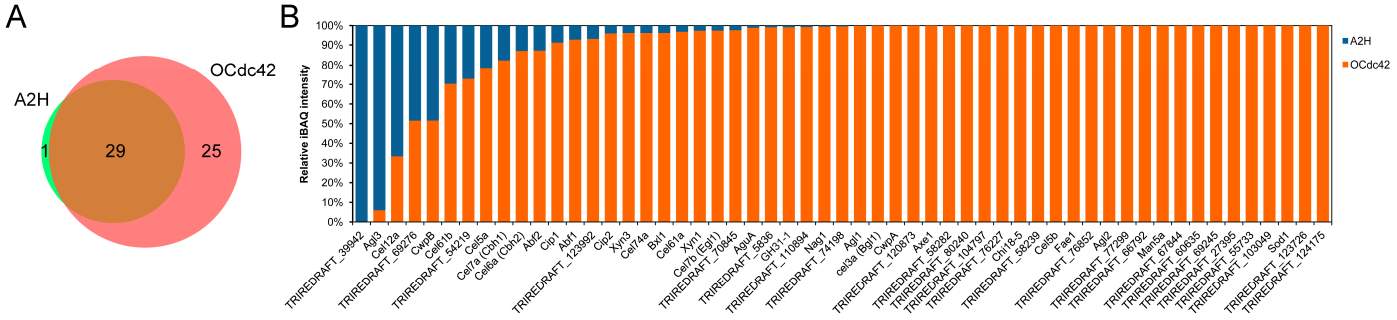

Figure 5. Comparative proteomic analyses between A2H and OCdc42 strains. (**A**) Comparison of secretomes from culture supernatants of A2H (lime) and OCdc42 strains (red). (**B**) Relative abundance of secreted proteins in the supernatants of cultures of A2H and OCdc42 strains. Detailed information about the abundance is shown in Table S2 in the Supplemental Material.

These observed changes are likely to play a significant role in enhancing the protein secretion capacity of the OCdc42 strain during submerged cultivation. It is widely accepted that protein secretion by filamentous fungi occurs primarily at the young hyphal tips [61]. This result was consistent with previous studies showing that the process of branching results in the emergence of more growing tips which are crucial for protein secretion [62].

3.5. Mechanism of Regulation of Morphogenesis of T. reesei via Rho GTPases

In addition, the expression levels of *cla4*, *ras2*, *ras1*, *spa2*, *rho1*, and *rac1* are also upregulated in the OCdc42 strain (Figure 6), indicating that Cdc42 is involved in multiple cellular processes. This result was consistent with a previous study suggesting that in *T. reesei*, Ras1 or Ras2 may interact with Cdc42 to regulate the process of filamentous growth [63]. Ras2 signals via the Cdc42 cascade to regulate cell elongation and cell adhesion, ultimately controlling the filamentous growth in *S. cerevisiae* [64]. By regulating the transcription level of *cdc42*, it can influence the transcription level of other Rho GTPases such as Cla4, Ras2, Ras1, Spa2, Rho1, and Rac1. The interaction between Cdc42 and other Rho GTPases in *T. reesei* may be a mutual cross-linking relationship rather than a hierarchical upstream-downstream response relationship.

Rho GTPases are crucial binary switches in signaling pathways that transmit signals from the external environment to the nucleus, regulating cell proliferation, growth, and differentiation in various eukaryotes, from humans to yeast [65]. The regulation of morphogenesis through signaling pathways, such as cAMP signaling or MAP kinase signaling, is of great interest. In the mutant with a deletion for TrRas2, it was observed that there was no change in cAMP levels on cellulose or glucose, indicating that the effect of TrRas2 on cellulase gene transcription was not dependent on the cAMP signaling pathway [63].

By studying *S. cerevisiae* as an analog, we identified homologs of the MAP kinase signaling components involved in filamentous growth in the *T. reesei* genome, including TrSte20

(TRIREDRAFT_104364), TrSte11 (TRIREDRAFT_4945), TrSte7 (TRIREDRAFT_75872), and TrSte12 (TRIREDRAFT_36543). Interestingly, we observed a 3.6, 2.9, 5.3, and 4.8-fold transcriptional upregulation of TrSte20, TrSte11, TrSte7, and TrSte12, respectively, in the OCdc42 strain compared to the parent strain A2H.

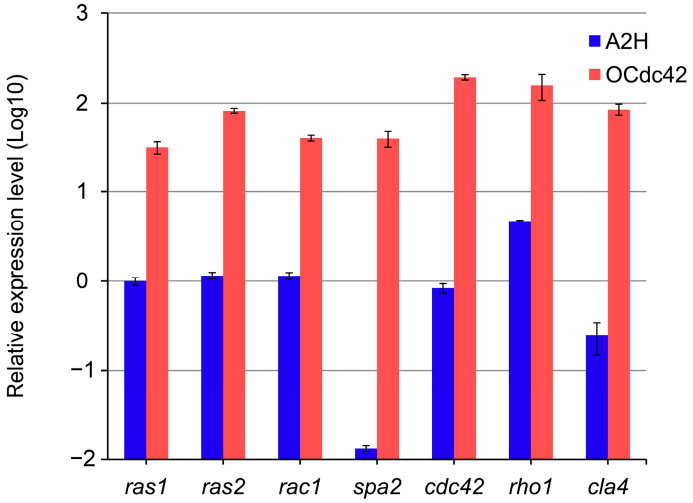

Figure 6. Expression levels of various branching-related genes in A2H and OCdc42 strains.

This suggests that the MAP kinase signaling pathway, rather than the cAMP signaling pathway, mediated by Cdc42 and other Rho GTPases in *T. reesei*, plays a role in the modulation of cellulase gene expression by regulating the transcription of transcription regulators for cellulase gene expression. As a molecular switch, Cdc42 responds to extracellular stimuli by transitioning from an inactive state (GDP-bound) to an active state (GTP-bound). This activation, in turn, regulates the polar growth of cells. Furthermore, Cdc42 positively regulates the MAPK pathway through positive feedback, thereby promoting hyphal formation [66].

4. Conclusions

Cellulase expression in filamentous fungi is a complex process in response to different environmental signals, with still unknown transcriptional regulatory mechanisms. Identification of novel factors affecting cellulase production is essential for improving the efficiency and yield of cellulase enzymes in *T. reesei*. Our study demonstrates that modulation of the Rho GTPase Cdc42 in *T. reesei* has a significant impact on both mycelial morphology and cellulase production. Deletion of *cdc42* resulted in impaired growth and reduced cellulase production, while overexpression of *cdc42* led to hyperbranched mycelium and enhanced cellulase production. This improvement in cellulase production was accompanied by the upregulation of cellulase genes and Rho GTPase genes. Additionally, secretome analysis reveals an increased abundance and diversity of extracellular proteins in the *cdc42*-overexpressing strain. These findings provide valuable insights into the functional role of Rho GTPases in *T. reesei* and present potential targets for engineering fungi to enhance the conversion of plant biomass in biorefineries. While this study has demonstrated the effect of *cdc42* overexpression on fungal characteristics, it is important to note that the underlying molecular processes and mechanisms have not been extensively explored. Future research should aim to delve deeper into elucidating the precise molecular mechanisms by which downstream factors of Cdc42 regulate fungal growth, hyphal branching, cellulase gene expression, and protein production in *T. reesei*.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation10010026/s1, Figure S1: GFP fluorescence-based sorting of *cdc42*-overexpressing protoplasts by flow cytometry; Table S1: Primers used in this study; Table S2: Proteome dataset of A2H and OCdc42 strains.

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