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The F-box protein gene *exo-1* is a target for reverse engineering enzyme hypersecretion in filamentous fungi

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Carbohydrate active enzymes (CAZymes) are vital for the lignocellulosebased biorefinery. The development of hypersecreting fungal protein production hosts is therefore a major aim for both academia and industry. However, despite advances in our understanding of their regulation, the number of promising candidate genes for targeted strain engineering remains limited. Here, we resequenced the genome of the classical hypersecreting Neurospora crassa mutant exo-1 and identified the causative point of mutation to reside in the F-box protein-encoding gene, NCU09899. The corresponding deletion strain displayed amylase and invertase activities exceeding those of the carbon catabolite derepressed strain Δcre -1, while glucose repression was still mostly functional in $\Delta exo-1$. Surprisingly, RNA sequencing revealed that while plant cell wall degradation genes are broadly misexpressed in $\Delta exo-1$, only a small fraction of CAZyme genes and sugar transporters are up-regulated, indicating that EXO-1 affects specific regulatory factors. Aiming to elucidate the underlying mechanism of enzyme hypersecretion, we found the high secretion of amylases and invertase in $\Delta exo-1$ to be completely dependent on the transcriptional regulator COL-26. Furthermore, misregulation of COL-26, CRE-1, and cellular carbon and nitrogen metabolism was confirmed by proteomics. Finally, we successfully transferred the hypersecretion trait of the exo-1 disruption by reverse engineering into the industrially deployed fungus Myceliophthora thermophila using CRISPR-Cas9. Our identification of an important F-box protein demonstrates the strength of classical mutants combined with next-generation sequencing to uncover unanticipated candidates for engineering. These data contribute to a more complete understanding of CAZyme regulation and will facilitate targeted engineering of hypersecretion in further organisms of interest.

F-box proteins | fungal biotechnology | *Neurospora crassa* | enzyme hypersecretion | CAZyme gene regulation

Filamentous fungi are important cell factories for diverse biotechnological products, such as enzymes, organic acids, and secondary metabolites. Compared to other systems, they stand out because of their unparalleled secretion capacity. Because of their environmental role as decomposers, fungi are the most valuable sources of carbohydrate active enzymes (CAZymes). They are therefore of steadily growing interest for various industrial applications, including biofuel production from plant biomass (1). Historically, the establishment of industrial CAZyme-hyperproducing strains, such as *Trichoderma reesei* (Rut-C30 and CL847) and *Myceliophthora thermophila* (cluster C1), was based on laborious random mutagenesis and extensive screening, which proved to be successful but frequently generated strains with substantial fitness defects (2–4). Current genome engineering techniques, including CRISPR-Cas9, allow strain construction by rational design,

Significance

Lignocellulose-based biorefinery relies on plant cell wall degrading enzymes. Current genome editing methods can create fungal enzyme hypersecreter strains by design. However, the identification of candidate genes for targeted engineering of this trait remains a bottleneck, and the necessity of specific inducer molecules further complicates production. By the resequencing of a classical hypersecreting *Neurospora crassa* mutant, we uncovered that mutation of a gene encoding an F-box protein (*exo-1*) causes inducer-independent hypersecretion of amylases, invertase, and pectinases. Systems biology and genetic studies of $\Delta exo-1$ shed light on the regulation of enzyme secretion in filamentous fungi, allowing targeted reverse engineering of industrially employed fungi, such as *Myceliophthora thermophila*, demonstrating the power of classical mutants in combination with modern sequencing and omics technologies.

The authors declare no competing interest.

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without the introduction of undesired background mutations (5, 6). The main roadblock to this approach is, however, our severely limited understanding of the regulatory networks controlling CAZyme production and protein secretion and, therefore, the identification of suitable targets for genetic manipulation and targeted strain engineering (7).

While the first principles of CAZyme regulation have started to unfold, our knowledge of the underlying regulatory networks remains fragmented. In brief, the mass production of plant cell wall-degrading enzymes in fungi commences with the liberation of specific inducer molecules from encountered lignocellulose. These signals are produced by the breakdown of plant cell wall polysaccharides (7) through "scouting" CAZymes, which are secreted in small amounts, even in absence of any inducer (8, 9). After uptake of these inducers into the hyphae by sugar transporters (10-12), signaling cascades are initiated, which remain mostly unknown but likely involve vast carbon source specific (de-) phosphorylation reactions (13). As a consequence, transcription factors (TFs) are activated that induce expression of specific gene regulons, including CLR-1/ClrA, CLR-2/ClrB (1, 14, 15), XLR-1/XlnR (16)/Xyr1 (17), and ARA-1/Ara1 (18, 19) for deconstruction of cellulose/hemicelluloses and PDR-1 (20)/ GaaR (21, 22), and PDR-2/RhaR (23) for pectin or COL-26/ AmyR/BglR for starch (24-27). These inducing pathways are competing with inhibitory factors, ensuring that energy is not wasted. For example, carbon catabolite repression (CCR) suppresses the production of CAZymes in the presence of glucose through the highly conserved TF CRE-1/CreA/Cre1 (28-30). Moreover, simultaneously occurring cellular demands and environmental cues need to be integrated, such as those regulating nutrient supply, metabolism, and development, which involve factors higher up in the regulation hierarchy. Examples of such regulators include VIB-1 for carbon scouting and vegetative incompatibility (18, 31, 32) or the Velvet family of regulators for light, sexual reproduction, and secondary metabolism (33). Notably, while new programs are activated to rewire cellular metabolic processes, the switch to a new environmental condition also demands the termination of preexisting programs to avoid interference. This reprogramming involves the targeted degradation of regulators, enzymes, and other proteins via the ubiquitin-proteasome system. Of central importance for this process are F-box domain-containing proteins, which recruit specific target proteins to the major E3 ubiquitin ligase, the Skp-Cullin-F-box (SCF) complex, for polyubiquitination. These proteins thereby affect a wide variety of cellular processes from nutrient assimilation to pathogenicity (34) and metabolism (35-37), including the wide domain repressor CRE-1/CreA/Cre1 itself (38). Overall, this regulatory and metabolic complexity severely hampers the prediction of suitable targets for the rational design of hypersecreting production strains.

For the genetic model organism, Neurospora crassa, a rich set of classical mutants exists, including enzyme-hypersecreting strains. For most of these isolates, the underlying genetic cause remains unknown. However, whole-genome sequencing has proven to be a powerful tool to identify the causative mutations in classical fungal mutant strains (39). We therefore hypothesized that the analysis of classical CAZyme-hypersecreting mutants will identify potentially unexpected targets for the rational design of hypersecreting fungi. Applying this strategy to the hypersecreting classical N. crassa mutant exo-1, we identified a premature nonsense mutation within the F-box protein-encoding gene NCU09899 (exo-1). Systems biology approaches revealed aberrant polysaccharide, sugar, organic acid, and amino acid metabolism in a $\Delta exo-1$ mutant, while CRE-1-dependent CCR was still mostly functional. Overexpression of amylases and invertases in the mutant is completely dependent on the regulator COL-26. By introduction of a $\Delta exo-1$ mutation into the industrial enzyme producer M. thermophila through CRISPR-Cas9, we demonstrate the translatability of our data to biotechnologically relevant species.

Overall, this study identified an F-box protein as a target for the rational design of CAZyme-overproducing fungal strains. The hypersecretion of several enzymes in the absence of CAZyme inducers is a particularly desirable trait for industrial enzyme and heterologous protein production, making F-box proteins the most promising targets for future strain development.

Results

Whole-Genome Sequencing of the exo-1 Strain Revealed the Causative Mutation. The exo-1 mutant (allele SF26) had been described as a hypersecretion strain of α -amylase, β -amylase, glucoamylase, invertase, pectinases, cellobiase, and trehalase, while the genetic basis remained unknown (40-46). Next-generation sequencing methods have enabled the association of mutant phenotypes with physical loci in the genome (47). A pilot mutant strain genome resequencing study in N. crassa successfully associated 16 mutant phenotypes with discrete genes (39). This method was scaled up, and over 500 N. crassa mutant strains, including exo-1 (48), were resequenced using this procedure. Comparative analysis among genome sequences from multiple N. crassa mutants identified a nonsense premature stop mutation in NCU09899 as being unique to sequences obtained from the exo-1 mutant strain. Therefore, we assigned the existing mutant to the open reading frame NCU09899, the exo-1 gene, which encodes a protein of 516 amino acids with a predicted F-box domain at its N terminus (amino acids 112 to 144) being highly related to the Frp1 (F-box protein required for pathogenicity) proteins described in Fusarium species (sp.) and Botrytis cinerea (SI Appendix, Fig. S1) (49–51). Blast analysis revealed high conservation of EXO-1 in the ascomycete classes Sordariomycetes, which also includes the biotechnologically highly relevant genus Trichoderma, and in the Leotiomycetes. Proteins with lower homology were identified in numerous species throughout most other classes of the filamentous ascomyete fungi (Pezizomycotina).

Since an *exo-1*-like background mutation had already been suggested for the *inositolless (inl)* mutant (46), we compared *inl* with *exo-1* and found highly similar protein secretion titers, SDS-polyacrylamide gel electrophoresis (PAGE) banding patterns, and invertase and amylase activity (*SI Appendix*, Fig. S2 *B–E*). When we resequenced the *exo-1* gene in *inl*, we found an insertion at base 717 from the start of the coding sequence, corroborating that an *inl*-unlinked secondary mutation in the *exo-1* gene exists in this specific *inl* strain (*SI Appendix*, Fig. S2A and Table S1). In addition, we identified an S11L missense mutation within the *exo-1* gene in two deoxyglucose-resistant (*dgr-2*) strains [(*dgr*(L1) and *dgr*(L5)] (48), with the serine being highly conserved.

Characterization of the $\Delta exo-1$ **Mutant Phenotype.** To further corroborate that the *exo-1* mutant phenotype was caused by the missense mutation in the locus of NCU09899, the wild-type (WT) *exo-1* gene was expressed at the *his-3* locus in the *exo-1* mutant background. Expression of the native *exo-1* gene fully complemented the amylase hypersecretion phenotype (Fig. 1A). Furthermore, a strain, in which the entire *exo-1* gene was deleted (strain $\Delta exo-1$), exhibited the classical *exo-1* mutant phenotype and secreted high amounts of CAZymes when grown consecutively for 4 d in medium containing either cellulose (Avicel), starch, or glucose (Fig. 1 *B–F*). The amount of secreted enzyme and enzyme activities were highly similar for both the *exo-1* and $\Delta exo-1$ strains.

N. crassa exo-1 is an ortholog to *Fusarium sp. frp1*, which exhibited severe growth reduction when complex substrates, soluble sugars, organic acids, amino acids, or sugar alcohols were used as carbon sources (51). However, the *N. crassa* $\Delta exo-1$ strain did not display any growth reduction on plates or race tubes under similar growth conditions (*SI Appendix*, Fig. S3).



Fig. 1. (*A–F*) Comparison of enzyme secretion and enzyme activities of *N. crassa* WT, $\Delta exo-1$ (Δe), exo-1 mutant (Ex), his^{3+} ::WTexo-1, exo-1 (Ex^{+}), $\Delta cre-1$ (Δc), and $\Delta exo-1/\Delta cre-1$ (n = 3). (*A*) amylase assay of strains directly inoculated in glucose medium for 4 d without shifting. (*B–F*) All strains were grown in Vogel's minimal medium with glucose for 48 h and then shifted to media containing either glucose or Avicel cellulose and soluble starch for 4 d. Enzyme secretion phenotypes were surveyed with SDS-PAGE (*B*), Bradford Assay (C), and the dinitrosalicylic acid (DNS) method for amylase activity (*D*), invertase activity (*E*), and CMCase activity (*F*). (*G–J*) CCR tests in exo-1 and $\Delta cre-1$ strain background. (*G*) Soluble starch agar growth assay of WT, exo-1, $\Delta exo-1$, $\Delta arc-1$, and $\Delta cre-1$, and $\Delta cre-1$ strains and $\Delta cre-1$, $\Delta cre-1$, $\Delta cre-1$, and $\Delta cre-1$ on 1% glucose concentrations (n = 3). (*H*) Relative growth of WT, $\Delta exo-1$, $\Delta cre-1$, and $\Delta exo-1/\Delta cre-1$ on 1% glucose plus 50 mM AA, compared to 1% glucose control (n = 6). Significance was determined by an independent two-sample *t* test of WT against all other strains. ** = P < 0.01, *** = P < 0.001, and n.d. = no growth detected. (*I* and *J*) Enzyme activities of the WT, $\Delta exo-1$, $\Delta cre-1$, and $\Delta exo-1/\Delta cre-1$ strains (n = 3). The strains were incubated for 48 h in 2% glycerol and then transferred to either NoC (*I*) or 2% sucrose (*J*) for another 24 h. Supernatants were used to determine enzyme activities through reducing end assays. Biomass dry weight was used to normalize the enzyme activities. Significance was determined by ANOVA followed by a post hoc Tukey's test (same letter = no significant difference and different letters = significant mean difference of P < 0.05). Error bars represent SD.

CCR Responses Are Partially Impaired in $\Delta exo-1$. The ortholog of EXO-1 in *Fusarium oxysporum*, Frp1, was described to work cooperatively with the TF Cre1, a main regulator for CCR (50). Deletion of *cre-1/cre1/creA* also leads to stronger expression of genes related to plant cell wall degradation in *N. crassa* and other fungi (52). In addition, it was reported that *dgr-2* mutants of *N. crassa*, in which we also identified a mutation in *exo-1*, are resistant to 2-deoxyglucose (2-DG), a commonly used glucose

analog to test functionality of CCR, suggesting that CCR is inactive in these strains. Also, hypersecretion had been reported for *exo-1* in the presence of galactose, which can induce CCR (44). On the other hand, enzyme secretion in *N. crassa exo-1* was described to commence only after glucose is depleted (42), arguing for functional CCR. To investigate this conundrum, we tested these findings in the $\Delta exo-1$ gene knockout strain. Initially, hypersecretion in the presence of sugars was tested on galactose, which had been reported to specifically induce pectinases in *exo-1* (44). We used the *N. crassa* WT and $\Delta exo-1$ strains to monitor the galactose consumption and activity of secreted enzymes in the culture supernatants (*SI Appendix*, Fig. S4). Surprisingly, enzyme secretion was not elevated in either strain in the presence of galactose, contrary to the earlier reports (44). Instead, the initiation of endopolygalacturonase, amylase, and arabinanase secretion coincided with the depletion of galactose to nonrepressing levels (at ~20 h; *SI Appendix*, Fig. S4). These data corroborate the reports by Gratzner and Sheehan (42) and suggest that at least certain parts of CCR are functional in $\Delta exo-1$.

Next, we examined the 2-DG tolerance of the *exo-1* and $\Delta exo-1$ strains on soluble starch plates in comparison to the deletion strain of the major CCR regulator, Δcre -1, as well as the WT (Fig. 1*G*). As a result, *exo-1* and $\Delta exo-1$ showed high tolerance to 2-DG, similar to Δcre -1, while WT growth was severely inhibited. These data suggest that certain branches of CCR are impaired in Δexo -1 and help to explain the reported phenotype of the *dgr*-2 strains (48).

An additional assay for CCR functionality uses allyl alcohol (AA), which is converted to toxic acrolein by carbon catabolite-repressed alcohol dehydrogenases (53). Derepressed strains, therefore, show a higher sensitivity to this alcohol analog, as seen for $\Delta cre-1$ (Fig. 1*H*; no growth detected). $\Delta exo-1$ was slightly more sensitive than the WT, but this branch of the CCR was clearly much more WT like than the one responsible for 2-DG sensitivity. A $\Delta exo-1/\Delta cre-1$ double deletion strain behaved like $\Delta cre-1$ (Fig. 1*H*).

To test the reported cooperative role of EXO-1 and CRE-1 (50) in more detail, we compared protein expression and enzyme activities of the Δcre -1 strain to the *exo*-1 and Δexo -1 mutants grown on Vogel's medium containing glucose, soluble starch, or Avicel cellulose. Although a higher protein secretion could be observed for the Δcre -1 strain, compared to the WT strain (Fig. 1B), the measured protein levels of Δcre -1 reached only ~50 to ~75% of the protein concentrations observed for the *exo*-1 and Δexo -1 strains (Fig. 1C). The enzyme activities also showed a significant difference between the Δcre -1 strain and the two *exo*-1 strains, as the Δcre -1 strain exhibited much lower invertase and amylase activities, compared to both the *exo*-1 and Δexo -1 strain (Fig. 1 *D*–*F*). The functions of EXO-1 and CRE-1 in regulating CAZyme production, therefore, appear to be different and at least in parts independent of each other.

To test functionality of the CRE-1-mediated branch of CCR in $\Delta exo-1$ specifically, we tested the $\Delta exo-1/\Delta cre-1$ double deletion strain in more detail (Fig. 1 I and J). This strain, as well as the single deletion mutants $\Delta exo-1$, $\Delta cre-1$, and the WT, was incubated on glycerol and transferred either to medium without carbon source (NoC) or sucrose. As described previously, the absence of any carbon source led to a strong expression of amylases and invertases in both the $\Delta exo-1$ and $\Delta exo-1/\Delta cre-1$ strains. However, the double deletion strain displayed significantly higher invertase activity than $\Delta exo-1$ in NoC medium (Fig. 11), indicating that the deletion of *cre-1* in the $\Delta exo-1$ background further improved invertase production. The $\Delta exo-1/\Delta cre-1$ strain also showed substantial amylase, CMCase, and invertase activity in high-sucrose medium, in which enzyme production in $\Delta exo-1$ was still repressed (Fig. 1J). Taken together, these results demonstrate that CRE-1-mediated CCR is still functional in $\Delta exo-1$, while other branches of CCR appear to be clearly affected.

 $\Delta exo-1$ Shows a Broad Misexpression of Genes Related to Carbon Source Utilization. To obtain a more global overview of the impact of the *exo-1* mutation on gene expression, we performed a transcriptomics analysis of $\Delta exo-1$ and WT by RNA sequencing (RNA-seq). To this end, the precultured strains were incubated

for 16 h in modified Vogel's medium either without any additional carbon source (NoC; "derepressed" state), with added glucose ("repressed" state), or with added Avicel ("induced" state).

Across all conditions, surprisingly, more genes were found to be down- than up-regulated in $\Delta exo-1$, compared to the WT (Fig. 2A and Dataset S1 and SI Appendix, Fig. S5). However, as expected, gene enrichment by Functional Categories (FunCat) showed that in the derepressed NoC condition, $\Delta exo-1$ displayed enriched starch metabolism (01.05.03.04), extracellular polysaccharide degradation (01.25.01), cellulose metabolism (01.05.03.05), and hemicellulose/pectin metabolism (01.05.03.06), compared to the WT (Dataset S1). On Avicel, expression patterns were more similar, but starch metabolism (01.05.03.04) was still significantly more induced in $\Delta exo-1$, compared to WT. Notably, categories comprising genes of the secretory apparatus, such as 20.09.16 (cellular export and secretion), were not found to be enriched in any condition tested (Dataset S1). These data confirm that the observed hypersecretion phenotype becomes active after release from CCR and is not based on a misexpressed secretory pathway per se.

To obtain a more detailed insight into the misregulation found in $\Delta exo-1$, we compared the sets of up- or down-regulated genes of this strain for the three different carbon source conditions (Fig. 2A). During derepressed conditions under starvation (Avicel and NoC together), 89 genes were found to be up-regulated in $\Delta exo-1$. In accordance with the observed secretion phenotype, genes encoding for factors involved in starch metabolism (01.05.03.04), sugar transport (20.01.03.01), and hemicellulose/ pectin metabolism (01.05.03.06) showed a significant enrichment in this group (Dataset S1). The specific transcriptional response of $\Delta exo-1$ to NoC (193 up-regulated genes) was broader, showing significant enrichment for extracellular polysaccharide degradation (01.25.01). While no category was significantly enriched regarding up-regulated genes on Avicel in $\Delta exo-1$ (140 genes), most down-regulated genes (186 genes) were related to extracellular polysaccharide degradation (01.25.01), hemicellulose/pectin metabolism (01.05.03.06), and cellulose metabolism (01.05.03.05.07), indicating that polysaccharide metabolism was nonuniformly affected by loss of *exo-1*. When incubated with glucose, $\Delta exo-1$ exhibited no significant enrichment in up-regulated (120 genes) or down-regulated genes (285 genes) for categories involved in polysaccharide degradation and metabolism.

A Specific Subset of CAZyme-Encoding Genes Is Positively Affected in *Lexo-1.* A hierarchical clustering of all CAZyme-encoding genes (236 genes) was performed with the RNA-seq data to determine with more resolution how the genes of the polysaccharide degradation apparatus are specifically affected in $\Delta exo-1$ (Fig. 2B) and Dataset S2). This analysis revealed 21 clusters; of which, most showed a weaker or similar gene expression in $\Delta exo-1$, compared to the WT on the same carbon sources. Only clusters C1, C11, and C21 contained genes significantly up-regulated in $\Delta exo-1$ (86 genes). Most genes in C1 (61 genes) were encoding CAZymes that were strongly up-regulated in $\Delta exo-1$ during starvation (NoC) but exhibited a reduced expression on Avicel and were WT like repressed under CCR in glucose (Dataset S2). We divided C1 into four subclusters, since a clear distribution of the genes to distinct sets of plant cell wall degradation functions was observed. Subcluster 1 contains many CAZymes related to cellulose degradation (26 genes), while subcluster 2 is composed mostly of genes encoding hemicellulases and some pectinases (14 genes). The third subcluster contains genes related to pectin degradation (seven genes) and the fourth subcluster showed a mixed distribution of CAZymes (14 genes). Cluster C11 comprised 18 genes with at least twofold up-regulation in $\Delta exo-1$ on both Avicel and NoC, compared to the WT. This cluster contained most CAZymes known to be highly secreted in exo-1, such as the glucoamylase gla-1 (NCU01517; confer Fig. 1B) and the invertase inv (NCU04265)



Fig. 2. Differentially gene expression analysis of the $\Delta exo-1$ strain compared to the WT strain in glucose (Glc), Avicel, and NoC medium (n = 3). (A) Venn diagram analysis of significantly up- or down-regulated genes (CuffDiff) of the $\Delta exo-1$ strain (≥ 10 fragments per kilobase of transcript per million mapped reads [FPKM] and minimum twofold change). Cluster analysis of differentially expressed CAZyme genes (B) and transporter genes (C) of the $\Delta exo-1$ strain compared to the WT strain in Glc, Avicel, and NoC medium (≥ 10 FPKM). (Scale bar indicates twofold change.) "C" represents clusters and "S" sub clusters. Categories in C were annotated with the TCDB. 2.A.1.1: sugar porters, 2.A.2: sucrose transporter, and 2.A.1.7: fucose–proton symporter.

but also some genes encoding cellulases and pectinases (Dataset S2). A double deletion strain of $\Delta exo-1$ and $\Delta gla-1$ was created to determine whether GLA-1 is one of the main secreted amylases, as predicted by our RNA-seq results. Indeed, the loss of the gla-1 gene completely abolished the amylase hypersecretion phenotype in the double mutant (*SI Appendix*, Fig. S6). The $\Delta exo-1/\Delta gla-1$ double deletion strain also displayed elevated invertase and CMCase activity on both Avicel and Switchgrass medium (*SI Appendix*, Fig. S6), indicating that the deletion of gla-1 relieved the secretion apparatus to some extent and opened up capacity for other secreted enzymes.

The last cluster, C21, encompassed genes that had a higher expression in Δexo -1 on glucose, compared to WT. However, most of these seven genes were either hypotheticals or not related to plant cell wall degradation. Thus, in total, only 79 out of 236 CAZyme genes displayed a strong up-regulation in Δexo -1 induced with Avicel and/or NoC over WT.

Deletion of exo-1 Leads to Increased Expression of Glucose and Xylose Transporters. Sugar transport reactions are tightly linked with plant cell wall degradation metabolism. To test if these reactions are affected by the loss of *exo-1*, 102 genes coding for putative sugar transporters were annotated using the Transporter Classification Database (TCDB) and clustered using the RNA-seq data (Fig. 2*C* and Dataset S3).

The transporter genes clustered into nine groups, but only five clusters (C1 and C3 to C6) contained genes directly annotated as sugar transporters (TCDB categories 2.A.1.1 and 2.A.1.7). As for the CAZyme genes, most of the transporter genes were either weaker or equally expressed in Δexo -1, compared to the WT on all tested conditions. However, clusters C5 and C6 showed several transporter genes that were highly up-regulated in Δexo -1 on Avicel and/or NoC, including the major low-affinity glucose transporter GLT-1 (NCU01633), the high-affinity glucose transporters HGT-1 (NCU10021) and HGT-2 (NCU04963), and the xylose transporter XYT-1 (NCU05627) (54, 55). These results demonstrate that the starch-related hyperderepression phenotype of Δexo -1 extends also to the level of the corresponding transporters, allowing the mutant to adjust uptake of glucose to the increased enzymatic activity.

Δ*exo-1* Amylase and Invertase Hypersecretion Is Completely Dependent on COL-26. Considering the strong (albeit nonexclusive) bias of the *exo-1* phenotype toward hyperderepression of starch-degrading enzymes, we hypothesized that the starch deconstruction regulator COL-26 might be directly or indirectly affected by the loss of EXO-1. By comparing our RNA-seq data to the previously published COL-26 regulon (25, 32), we found an obvious overlap (*SI Appendix*, Fig. S7*A*), which included genes such as *gla-1*, *inv*, and *vib-1* (NCU03725). To further test our hypothesis, we generated a Δ*exo-1*/Δ*col-26* double deletion strain and tested growth on 2% maltose (*SI Appendix*, Fig. S7*B*). Both Δ*col-26* alone and Δ*exo-1*/ Δ*col-26* showed a strong growth defect on maltose unlike the WT and Δ*exo-1*. The same four strains were then grown in Vogel's acetate medium for 48 h and shifted to NoC medium. Enzyme assays revealed that deletion of $\Delta col-26$ in the $\Delta exo-1$ background completely reverted amylase and invertase secretion to WT levels, while endopolygalacturonase and arabinanase activities remained similar to $\Delta exo-1$ (*SI Appendix*, Fig. S7C). These results demonstrate that amylase and invertase hypersecretion in $\Delta exo-1$ is completely dependent on COL-26.

The $\Delta exo-1$ Proteome Shows Changes in Carbon and Nitrogen Metabolism. As an F-box protein, EXO-1 could act as part of the ubiquitination machinery regulating protein turnover, as previously suggested for its ortholog in *F. oxysporum* (56). To test for aberrant protein abundances in $\Delta exo-1$, compared to WT, we designed a proteomics experiment in which both strains were switched to either NoC or glucose medium and incubated for 1 or 16 h. Protein profiles were compared for both strains in repressed (glucose) and derepressed (NoC) conditions (Dataset S4).

Based on our findings on the role of COL-26, we first looked for changes in the abundance of this regulator. The protein level of this TF was significantly increased in $\Delta exo-1$, compared to the WT on both glucose and NoC after 1 h (*SI Appendix*, Fig. S8B). In contrast, CRE-1 abundance was 3.6-fold higher in $\Delta exo-1$, compared to the WT after a 1 h incubation with glucose, while there were no significant changes on NoC (*SI Appendix*, Fig. S8A). These data further support a link between COL-26 and EXO-1. Furthermore, the significantly increased CRE-1 levels on glucose might indicate a compensatory action of this TF directly or indirectly caused by the loss of EXO-1.

Proteins with significantly altered abundance (p-val < 0.05, twofold change; Dataset S4) in $\Delta exo-1$, compared to the WT, were used for a FunCat enrichment analysis to identify potentially affected pathways (*SI Appendix*, Fig. S8C). In the 16-h glucose condition (71 proteins), proteins belonging to categories related to nitrogen and amino acid metabolism (01.01.09) were upregulated in $\Delta exo-1$. Furthermore, polysaccharide metabolism (01.05.03) and metabolism of vitamins, cofactors, and prosthetic groups (01.07) were enriched, while proteins with reduced abundance (82 proteins) belonged to diverse and rather unspecific categories. After 16 h of starvation (NoC; 104 proteins), several categories were up-regulated in the mutant dealing with RNA processing (11.04) but also starch (01.05.03.04) and polysaccharide metabolism (01.05.03). Intriguingly, among the significantly down-regulated proteins in $\Delta exo-1$ on NoC (137 proteins), mostly carbon metabolism categories were enriched, such as C compound and carbohydrate metabolism (01.05); lipid, fatty acid, and isoprenoid metabolism (01.06); and amino acid metabolism (01.01) but also proteasomal degradation (ubiquitin/proteasomal pathway) (14.13.01.01). Significant down-regulation was also detected for the ubiquitin transferases NCU00272 (*cul4*), NCU03947, NCU06372, NCU01225 (*uce-13*), and NCU10477 (*uce-5*). Taken together, these data indicate that the loss of EXO-1 affects broader cellular carbon and nitrogen metabolism, as well as the targeted protein degradation machinery.

Engineering CAZyme Hypersecretion in Filamentous Fungi Using exo-1. An important question was whether it is possible to reverse engineer the $\Delta exo-1$ hypersecretion phenotype into related fungi, preferentially with industrial relevance. To this end, we chose the filamentous ascomycete M. thermophila (American Type Culture Collection 42464), since this fungus possesses the exo-1 homolog, JGI: Spoth2|2296508 (57), and is already employed in biotechnological applications (4). The exo-1 homolog, Mtexo-1, was successfully deleted using a recently developed CRISPR-Cas9 system (6) (Fig. 3). Shift experiments of three independent $\Delta M texo-1$ isolates (M1 to M3) and M tWT from glucose medium to Avicel, starch, or glucose medium confirmed a phenotype with high similarity to the one observed in N. crassa. The $Mt\Delta exo-1$ strains exhibited up to threefold increased protein secretion and also up to threefold amylase activity on inducing and noninducing media after 4 d of incubation, while not displaying any aberrant growth phenotype on these or several other tested carbon sources (Fig. 3 and SI Appendix, Fig. S9). Interestingly, the amount of total extracellular protein increased only moderately in $Mt\Delta exo-1$, when grown on Avicel, and was lower than during the growth on glucose or starch, suggesting that EXO-1 is equally important for



Fig. 3. (*A*) Schematic of *exo-1* deletion mediated by CRISPR-Cas9 in *M. thermophila*. (*B*) SDS-PAGE of secreted protein of $Mt\Delta exo-1$ and WT strain MtWT pregrown on 2% glucose medium for 48 h and then shifted to 2% starch (Lane 1 to 2), 2% glucose (Lane 3 to 4), and 2% Avicel (Lane 5 to 6) for 4 d incubating, respectively. (*C* and *D*) Assays for protein concentration and amylase activities of culture supernatants are displayed in the same order as described for the SDS-PAGE. Error bars represent SD (n = 3). PAM, protospacer adjacent motif.

the regulation of enzymes involved in starch degradation in this thermophilic fungus like in *N. crassa*.

Discussion

In this study, mutations in the F-box protein gene exo-1 were identified to be causative for the long-known amylase, invertase, and pectinase hypersecretion phenotype of the classical N. crassa mutants exo-1, inl, and dgr-2 (42, 46, 48). These findings were transferred to the biotechnological important fungus M. thermophila and a hypersecreting strain was constructed by targeted mutation of the exo-1 homolog. While F-box proteins have been found to be involved in the regulation of CCR and xylanase secretion (36), this report links them to the hypersecretion of specific enzymes in the complete absence of CAZyme inducers, a highly desirable trait for enzyme production. Although a strong bias for starch-degrading enzymes exists in exo-1, the overall variety of misregulated enzymes in the absence of inducers suggests that EXO-1 affects multiple regulatory pathways for CAZyme secretion (58, 59). A likely explanation for this broad, albeit not global, effect is that EXO-1 might act through the stabilization/ destabilization of regulatory proteins. The correlation between hypersecretion of specific enzymes and up-regulation of the respective genes, being most evident for the glucoamylase GLA-1, suggests that this regulation takes place on the level of gene transcription. Our data strongly suggest that this transcriptional control is mediated through COL-26 but might further include CRE-1 and VIB-1 (25, 28, 32). This control mechanism is well known from plants [e.g., auxin and jasmonate signaling; (60, 61)] to fungi [e.g., sulfur assimilation; (35)], and these regulators in turn have their own broad regulons. COL-26, for example, appears to mediate correct integration of carbon and nitrogen metabolism in addition to its function as the major starch regulator (25), which might also explain the impaired amino acid metabolism found in our proteomics analysis.

F-box proteins can execute their regulatory function by recruiting specific proteins to the SCF complex, which mediates the ubiquitination and subsequent degradation of the target through the proteasome (62, 63). We hypothesize that EXO-1 mediates the adaptation to a new carbon source, through the activation and inactivation of specific regulatory factors, in order to remove the preexisting cellular program and to switch on the appropriate metabolism. In the plant pathogenic fungus F. oxysporum, loss of the exo-1 ortholog frp-1 not only affected pathogenic growth but also resulted in broad metabolic effects (49-51, 56). Fo Afrp-1 displayed compromised growth on pectin, sugar alcohols, amino acids, and organic acids. In contrast, no growth defects were observed in the N. crassa $\Delta exo-1$ mutant on those carbon sources, and the mutant strain grows even better than the WT on starch, sorbitol, and glutamate (*SI Appendix*, Fig. S3). Similarly, deletion of *frp1* in the plant pathogenic gray mold *B. cinerea* did not lead to any growth defects as well (51). Together, these observations suggest that the conserved exo-1-like F-box proteins are wired into different regulatory pathways in the various species. While their general function is likely the same, their targets might differ depending on the specific biology of the fungal species.

Our systems biology and genetic tests uncovered several so far unknown aspects of the *exo-1* phenotype. Surprisingly, only a specific subset of CAZyme genes was highly expressed in $\Delta exo-1$ under nonrepressing conditions (Avicel and NoC), and most CAZyme- and transporter-encoding genes showed lower expression in $\Delta exo-1$, compared to the WT. Among these genes were enzymes involved in starch and pectin degradation but also sugar transporters, such as *glt-1*, *hgt-1*, *hgt-2*, and *xyt-1* (Fig. 2). Particularly the concomitant hyperderepression of starch-degrading enzymes and glucose transporters indicates that coregulated functional groups are misregulated, supporting the notion that EXO-1 likely acts through transcriptional regulators, such as COL-26. This hypothesis is further strengthened by the substantially overlapping regulons, the finding that the $\Delta exo-1$ amylase and invertase hypersecretion was completely COL-26 dependent, and the persistently higher abundance of COL-26 in $\Delta exo-1$. Intriguingly, we also identified a potentially causative mutation of the *col-26* gene in the classical strain *dgr-1* (Fungal Genetics Stock Center [FGSC] No. 4325) (*SI Appendix*, Table S1). $\Delta col-26$ displays 2-DG resistance (32) similar to *dgr-1*, which itself is phenotypically highly related to the *exo-1* allele *dgr-2* (48), adding yet another genetic connection between COL-26 and EXO-1.

Similarly, VIB-1 was previously found to be essential for several aspects of the *exo-1* phenotype, since the *exo-1*/ Δvib -1 strain exhibited strongly reduced enzyme secretion in maltose medium, compared to *exo-1*, while displaying better growth and glucoamylase secretion than Δvib -1 alone (64). Overexpression of VIB-1, on the other hand, enhanced cellulase secretion in *T. reesei* Rut-C30 (65), which might therefore also represent a target for further increasing of enzyme secretion in Δexo -1 by additional engineering.

Improved secretion is also observed by mutants of the main regulator of CCR, *cre-1* (52). While $\Delta exo-1$ clearly shows a (hyper-) derepressed phenotype and is resistant to 2-DG [similar to the *dgr-2* alleles; (48)], our proteomic analysis showed that CRE-1 appears to be stabilized under certain conditions in $\Delta exo-1$. Enzymatic and sugar consumption assays, as well as the test for AA sensitivity and RNA-seq analysis, confirmed that, overall, CCR is still functional under repressing conditions (presence of glucose). When deleted, the combination of $\Delta cre-1$ and $\Delta exo-1$ largely removed CCR, leading to the secretion of amylases and invertase also under repressing conditions. This phenotype is highly desirable from a biotechnological point of view and, furthermore, demonstrates that an arm of CCR is affected in $\Delta exo-1$ that is not CRE-1 mediated.

Overall, the collected data suggest that EXO-1 is involved in the metabolic adaptation upon the depletion of extracellular carbon sources and beginning starvation, including the correct adjustment of starch, pectin, and hemicellulose metabolism, as well as the regulation of amino acid, organic acid, lipid, and alcohol metabolism. Based on the gathered information, we propose a model in which EXO-1 destabilizes COL-26 (or a putative COL-26 activator) under derepressed conditions (Fig. 4). Under



Fig. 4. A model of EXO-1 function. The F-box protein EXO-1 targets either the amylase and invertase regulator COL-26 directly or a putative regulator thereof for degradation, which leads to moderate levels of amylase and invertase under starvation conditions. Deletion of *exo-1* causes high secretion of amylase and invertase and repression of (most) cellulases. CCR, as imposed through CRE-1, counteracts enzyme secretion caused by *exo-1* deletion in high-glucose conditions, while removal of *cre-1* causes substantial enzyme secretion also in the presence of glucose.

APPLIED BIOLOGICAL SCIENCES cellulolytic and starving conditions, EXO-1 likely regulates the abundance of those respective proteins to fine-tune enzyme secretion. At the same time, CCR is still functional and can prevent enzyme hypersecretion in $\Delta exo-1$ in excess of glucose. In addition to the stabilization of CRE-1, the increased abundance of COL-26 in $\Delta exo-1$ could also explain the observed downregulation of most CAZyme genes, since COL-26 and its homolog AmyR are known to repress cellulases while activating amylases (5). The COL-26 homolog BglR was further found to up-regulate the expression of the β -glucosidase genes bgl1 and bgl2 in T. reesei (26). Using the transcriptome data generated in this study, we were able to confirm that the putative orthologs of those two T. reesei genes, gh1-1 (NCU00130) and gh3-4 (NCU04952), were the most strongly up-regulated β -glucosidases in $\Delta exo-1$ versus WT in the NoC condition, compared with other GH1 and GH3 enzymes (Dataset S5), further supporting the hypothesis of an activation of COL-26 in absence of EXO-1.

Finally, enzyme hypersecretion by deletion of exo-1 was successfully reverse engineered into the industrially relevant fungus M. thermophila via CRISPR-Cas9, showing applicability of this strain engineering approach. This study, therefore, identified and used an F-box protein as a target for the rational design of enzyme-hypersecreting strains. Since exo-1 orthologs are conserved in most Ascomycete fungal lineages, including industrially employed fungi and plant pathogens, a comprehensive description of the role and function of this protein will be of great academic and applied interest. We hypothesize that exo-1 homologous genes, but also other F-box protein-encoding sequences, are highly promising targets for future strain improvement. Fungal genomes usually contain several F-box protein-encoding genes. It will, therefore, be highly relevant and most promising to identify their specific interaction partners, in order to map out their individual or combined potential for different, specific biotechnological applications.

Materials and Methods

For a detailed description, please see *SI Appendix, SI Materials and Methods*. Mutants were created via transformation or crossing and genotyped via PCR. Fungal cultures were grown on modified Vogel's minimal medium (66) [NH₄NO₃ replaced against 25 mM (NH₄)₂SO₄ and 32.5 mM sodium citrate;

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SI Appendix, Figs. S10 and S11], which decreased variability of growth and protein secretion across biological replicates and experiments.

For enzyme studies, the Bradford Protein Assay (67) with the bovine gamma globulin standard was used for enzyme quantification and SDS-PAGE with 8 to 16% Mini-PROTEAN Precast Protein Gels for protein profiling. The dinitrosalicylic acid (68) assay was used to compare amylase, invertase, CMCase, and trehalase activities of different fungal strains. Similarly, a modified version of the Megazyme carbohydrate reducing ends assay (Megazyme) using 4-hydroxybenzhydrazide was used to determine invertase, amylase, and arabinanase enzyme activities. Endopolygalacturonase activity was assessed, as described previously (69).

RNA-seq was performed with the WT (FGSC No. 2489) and $\Delta exo-1$ (FGSC No. 19860) strains grown in 1× modified Vogel's glucose medium for 48 h and shifted to 1× modified Vogel's plus either 2% p-glucose, 0.5% cellulose (Avicel), or no carbon source for 16 h. The TruSeq Stranded Total RNA Prep kit (Illumina) was used for library preparation and subsequent sequencing on a MiSeq System (Illumina). For further analyses, a twofold expression threshold was applied to these genes, and genes with an expression of less than 10 fragments per kilobase of transcript per million mapped reads were removed from the analysis. For proteomics, mycelium samples were lysed with a TissueLyzer and extracted with 8 M urea with subsequent dilution for trypsin digestion for mass spectrometry.

Data Availability. All study data are included in the article and/or supporting information.

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