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The transcriptional activator ClrB is crucial for the degradation of soybean hulls and guar gum in *Aspergillus niger*

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ABSTRACT

Low-cost plant substrates, such as soybean hulls, are used for various industrial applications. Filamentous fungi are important producers of Carbohydrate Active enZymes (CAZymes) required for the degradation of these plant biomass substrates. CAZyme production is tightly regulated by several transcriptional activators and repressors. One such transcriptional activator is CLR-2/ClrB/ManR, which has been identified as a regulator of cellulase and mannanase production in several fungi. However, the regulatory network governing the expression of cellulase and mannanase encoding genes has been reported to differ between fungal species. Previous studies showed that *Aspergillus niger* ClrB is involved in the regulation of (hemi-)cellulose degradation, although its regulon has not yet been identified. To reveal its regulon, we cultivated an *A. niger* Δ*clrB* mutant and control strain on guar gum (a galactomannan-rich substrate) and soybean hulls (containing galactomannan, xylan, xyloglucan, pectin and cellulose) to identify the genes that are regulated by ClrB. Gene expression data and growth profiling showed that ClrB is indispensable for growth on cellulose and galactomannan and highly contributes to growth on xyloglucan in this fungus. Therefore, we show that *A. niger* ClrB is crucial for the utilization of guar gum and the agricultural substrate, soybean hulls. Moreover, we show that mannobiose is most likely the physiological inducer of ClrB in *A. niger* and not cellobiose, which is considered to be the inducer of *N. crassa* CLR-2 and *A. nidulans* ClrB.

1. Introduction

Plant biomass is the most abundant carbon source on Earth and consists mainly of plant cell wall polysaccharides (cellulose, hemicellulose and pectin), and the aromatic polymer lignin. Various plant substrates are used for applications in food, pharmaceutical, textile or paper & pulp industry (de Vries et al., 2020). One such substrate is guar gum (GG), an abundant biopolymer found in *Cyamopsis tetragonolobus* seed endosperm (Thombare et al., 2016), which is used as a thickener, stabilizer agent and additive in pharmaceutical, paper, textile or food industries (Mudgil et al., 2014). Its main component is galactomannan (Malgas et al., 2015), a polymer which is also abundantly present in soybean hulls (SBH) (Whistler and Saarnio, 1957). Soybean is cultivated in large amounts worldwide (up to 240 million tons per year), producing approximately 20 million tons of SBH per year as a major by-product (Liu and Li, 2017). Soybean hulls are used as animal feed, in the treatment of waste water or as dietary fibers, and it is a relevant feedstock for biotechnological applications (Liu and Li, 2017).

To produce valuable compounds from plant biomass, a broad set of hydrolytic and oxidative enzymes are required to degrade its main polymers. Filamentous fungi are efficient plant biomass degraders due to their extensive set of Carbohydrate Active enZymes (CAZy, https://www.cazy.org) (Lombard et al., 2014) they can secrete. One of the most important filamentous fungi used in biotechnology is *Aspergillus niger*, which is well known for its ability to produce a broad range of enzymes required for the degradation of plant polysaccharides (de Vries and Visser, 2001). Transcription Factors (TF) play an essential role in the regulation of gene expression, controlling the production of a set

Abbreviations: GG, guar gum; SBH, soybean hulls; CAZyme, carbohydrate active enzyme; TF, transcription factor; MM, minimal medium; CM, complete medium; GH, glycoside hydrolase; CE, carbohydrate esterase.

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of enzymes that matches the polysaccharides that are present in the environment of the fungus. Several fungal TFs related to plant biomass degradation have been described (Benocci et al., 2017). Some transcription factors are associated with the degradation of specific substrates, such as GaaR (Alazi et al., 2016), which is the key regulator of pectin degradation (Kowalczyk et al., 2017). Other TFs control the degradation of a broader set of substrates. For instance, XlnR (van Peij et al., 1998b) controls the expression of cellulolytic, xylanolytic, mannanolytic and xyloglucanolytic genes (de Vries et al., 1999; Hasper et al., 2002; van Peij et al., 1998a), while AraR (Battaglia et al., 2011) plays an important role in hemicellulose and pectin degradation (Kowalczyk et al., 2017).

In *N. crassa*, the regulation of cellulose degradation is controlled by two TFs, CLR-1 and CLR-2 (Coradetti et al., 2012). ClrA, the ortholog of CLR-1, has also been identified in *Aspergillus nidulans* (Coradetti et al., 2012) and *A. niger* (Kun et al., 2021; Raulo et al., 2016) and was reported to be involved in cellulose degradation in these species. Orthologs of CLR-2 have been identified in *A. nidulans* (ClrB) (Coradetti et al., 2013, 2012), *A. niger* (ClrB) (Raulo et al., 2016), *A. oryzae* (ManR) (Ogawa et al., 2012) and *Penicillium oxalicum* (ClrB) (Li et al., 2015) that showed conserved function with respect to cellulose degradation, although *A. oryzae* ClrB (ManR) had been first reported as a TF regulating mannan degradation (Ogawa et al., 2012). A later study also showed the involvement of *N. crassa* CLR-2 in the regulation of mannan utilization (Samal et al., 2017). A detailed study of the ClrB ortholog from *A. nidulans* showed that ClrB controls the expression of several mannanolytic genes such as the β-mannosidase *mndB* or the endomannanase *manB* (Li et al., 2016). However, it was reported that the induction of mannanases in *A. nidulans* is more likely controlled by a ClrB paralogue, AN6832 (Li et al., 2016), which is not present in *A. oryzae* or *A. niger* (Kunitake and Kobayashi, 2017). The induction and/or the role of CLR-2/ClrB/ManR orthologs within the regulatory network of (hemi-)cellulose degradation appears to be strikingly different (Coradetti et al., 2013, 2012; Kunitake and Kobayashi, 2017). More recently, a homolog of ClrB (TclB2) has been identified in *Talaromyces cellulolyticus* that is involved in the regulation of mannan degradation, but not cellulose or xylan degradation (Fujii et al., 2021). A less conserved homolog of ClrB (CLR2) has also been described in *Trichoderma reesei*, but results showed only a minor influence of CLR2 on cellulase regulation in this species (Häkkinen et al., 2014). In contrast, light-dependent control of xylanase and pectinase encoding genes has been associated with CLR2 in *T. reesei* (Beier et al., 2020). Even though the involvement of *A. niger* ClrB in cellulose utilization has been previously reported (Kun et al., 2021; Raulo et al., 2016), it is not fully known to which extent it may also control the degradation of mannan or other plant polysaccharides. Better understanding of the regulon of ClrB in the industrially relevant filamentous fungus *A. niger*, allows for the generation of strains with improved ability to produce enzymes necessary for plant biomass degradation on an industrial scale.

In this study, we assessed the involvement of ClrB in the degradation of SBH and GG by *A. niger*. We showed that ClrB is involved in the degradation of cellulose, galactomannan and xyloglucan in *A. niger*. Moreover, we showed that mannobiose is most likely the inducer of ClrB in this species, despite cellobiose being the inducer of ClrB in *A. nidulans*, suggesting distinct activation of (hemi-)cellulolytic systems within closely related ascomycetes.

2. Materials and methods

2.1. Strains, media and growth conditions

Fungal strains used in this study were derived from the *A. niger* CBS 138852 (*cspA1*, *pyrG*$^{-}$, *kusA*::*amdS*) strain (Meyer et al., 2007). All deletion mutants used in this study were previously generated by using the CRISPR/Cas9 system (Kun et al., 2021) and were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute under strain numbers indicated in **Table S1**. Strains were grown at 30 °C in *Aspergillus* Minimal Medium (MM) or Complete Medium (CM) (de Vries et al., 2004) supplemented with 1 % D-glucose and 1.22 g/L uridine (Sigma Aldrich). Growth profiles were performed using *Aspergillus* MM containing 25 mM mono-/disaccharides or 1 % polysaccharides/crude substrates.

The SBH used in this study was washed as previously reported (Kun et al., 2021). The washed supernatant-free pellet was resuspended in MM with 1 % final concentration for both growth profiles and liquid culturing. All media were supplemented with 1.22 g/L uridine. All growth profile plates were inoculated in duplicates with 10^3 spores and incubated at 30 °C for up to 14 days. Pictures were taken after 5, 6, 7, 8, 10 and 14 days of incubation and were evaluated by visual inspection, considering the colony diameter, mycelial density and sporulation.

2.2. Transcriptomic analysis

For transcriptomic analysis, freshly harvested spores were pre-grown in CM containing 2 % D-fructose and 1.22 g/L uridine for 16 h at 30 °C in a rotary shaker at 250 rpm. The mycelium was harvested by filtration through sterile cheesecloth, thoroughly washed with MM, and approximately 2.5 g (wet weight) mycelium was transferred to either 10 mL MM containing 2 mM cellobiose (Acros Organics) or mannobiose (Megazyme), or to 50 mL MM containing 1 % SBH or GG (**Table S2**). Mycelia were harvested for RNA isolation after 1 h incubation at 30 °C in a rotary shaker at 250 rpm in case of 2 mM mannobiose/cellobiose cultures, and after 2, 8 and 24 h incubation at the same condition in case of 1 % SBH/GG cultures. Mycelia were frozen in liquid nitrogen followed by storage at −80 °C until further use. Samples were collected in biological triplicates. The transcriptomes of the parental and mutant strains were analyzed using RNA-seq. RNA isolation, purification and quantitative and qualitative evaluation was performed as previously described (Garrigues et al., 2022).

Purification of mRNA, synthesis of cDNA library and sequencing were performed at the Joint Genome Institute (JGI). Plate-based RNA sample prep was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using Illumina's TruSeq Stranded mRNA HT sample prep kit utilizing poly-A selection of mRNA following the protocol outlined by Illumina in their user guide: https://support.illumina.com/sequencing/sequencing_kits/truseq-stranded-mrna.html, and with the following conditions: total RNA starting material was 1 μg per sample and 8 cycles of PCR was used for library amplification. The prepared libraries were then quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed and the pool of libraries was then prepared for sequencing on the Illumina NovaSeq 6000 sequencing platform using NovaSeq XP v1 reagent kits, S4 flow cell, following a 2x150 indexed run recipe. The processing of raw fastq reads and evaluation of raw gene counts were performed as previously described (Garrigues et al., 2022). Three biological replicates were generated and sequenced for each condition. Two individual samples were discarded from further analysis due to their poor sequencing quality.

Differentially expressed genes (DEGs) were detected using the R package DESeq2 (Love et al., 2014). Transcripts were considered differentially expressed if the DESeq2 fold change of the Δ*clrB* mutant strain compared to the control was > 2 (upregulation) or < 0.5 (downregulation) and *p*adj < 0.01 and at least one of the two expression values was FPKM > 20.

Heat maps for gene expression data visualization were generated using the "gplots" package of R software, with the default parameters: "Complete-linkage clustering method and Euclidean distance". The data used for the generation of heat maps are shown in **Data S1**. Genes with an expression value of FPKM < 20 in each sample were considered lowly expressed and were excluded from the analysis.

2.3. Binding site analysis

The ClrB consensus binding site was identified by using the online MEME version 5.3.3 tool (Bailey and Elkan, 1994) on the promoter regions of a set of 45 downregulated genes in the Δ*clrB* strain grown on SBH or GG. Promoter sequences were obtained from the JGI MycoCosm database (https://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html). Motif discovery mode was set to discriminative mode, using the promoter regions of all genes excluding the promoters of the 45 primary genes as control. Motif length was restricted to 10–18 nucleotides and specified the occurrences to zero or to one per sequence.

Binding site analysis was performed using the RSAT online tool (Thomas-Chollier et al., 2008) (https://rsat-tagc.univ-mrs.fr/rsat/dna-pattern_form.cgi) as previously reported (Kun et al., 2021). The presence of binding sites in the promoter regions of putatively ClrB-regulated genes are indicted in **Table S3**.

3. Results and discussion

3.1. The deletion of ClrB strongly reduces Aspergillus niger growth on soybean hulls and guar gum

To evaluate the contribution of ClrB to the utilization of SBH in *A. niger*, we performed a growth profile on this crude substrate, as well as on several major constituent mono- and polysaccharides, and GG (Fig. 1). GG is mainly composed of galactomannan, while SBH contains significant amounts of cellulose, galactomannan, pectin, xylan and xyloglucan (Aspinall et al., 1966; Karr-Lilienthal et al., 2005). The sugar composition of SBH and GG are indicated in **Table S2**.

Growth on GG was strongly reduced in the *clrB* deletion mutant (Fig. 1), indicating a large contribution of ClrB towards the degradation of galactomannan by *A. niger*. Growth on D-mannose was not affected in the Δ*clrB* strain, indicating little or no effect on the regulation of D-mannose transport or metabolism. Surprisingly, while SBH contains diverse polysaccharides, the deletion of *clrB* also resulted in strongly reduced growth on SBH. This suggests that ClrB is involved in the degradation of several major SBH components, such as cellulose, galactomannan and xyloglucan. Previous studies reported the involvement of ClrB in cellulose degradation in *A. niger* (Kun et al., 2021; Raulo et al., 2016), which correlates with abolished growth on cellulose in our study (Fig. 1). However, no growth reduction was observed on cellulose for an *A. oryzae manR* disruption mutant, despite its involvement in cellulose utilization (Ogawa et al., 2013). These results demonstrate differences in the extent of cellulase regulation by ClrB or the involvement of other cellulolytic regulators between these two species.

Interestingly, growth on cellobiose was not affected by the deletion of *clrB* (Fig. 1). Growth reduction on this substrate was observed in *A. nidulans* (Coradetti et al., 2012), but not in *N. crassa* (Coradetti et al., 2012), also highlighting differences in the role of ClrB in these species.

A transient growth reduction was observed on xyloglucan for the *A. niger* Δ*clrB* mutant, showing partial growth recovery after 14 days of growth (Fig. 1). The slow growth on xyloglucan may be related to the involvement of ClrB in the regulation of xyloglucanases, such as the Glycoside Hydrolase Family 12 (GH12) enzyme EglA (Kun et al., 2021), which is also supported by transcriptome data (see next sections). No growth reduction was observed on xylan or pectin, or on some of the (major) constituent sugars, D-xylose, L-arabinose, L-rhamnose and D-galacturonic acid (Fig. 1). The presence of xylan and pectin in SBH could only partially support growth of the *clrB* deletion strain, possibly because it has reduced access to xylan and pectin in SBH.

The crucial role of ClrB in the degradation of SBH and GG was also confirmed by an additional growth profile involving combinatorial deletions of major (hemi-)cellulolytic TFs XlnR, AraR, ClrA and ClrB that were previously generated (Kun et al., 2021). Our results showed that the Δ*clrB* single deletion strain, as well as each combinatorial deletion mutant carrying the deletion of *clrB* more strongly reduced growth on the test substrates compared to any other deletion strain (**Figure S1**). Moreover, the Δ*clrB* strain showed an even stronger growth reduction than the Δ*xlnR*Δ*araR*Δ*clrA* triple mutant, confirming that ClrB has a dominant role in the regulation of SBH and GG degradation. The important role of ClrB in the degradation of both SBH and GG could (mainly) be associated with the impaired ability of the *clrB* deletion

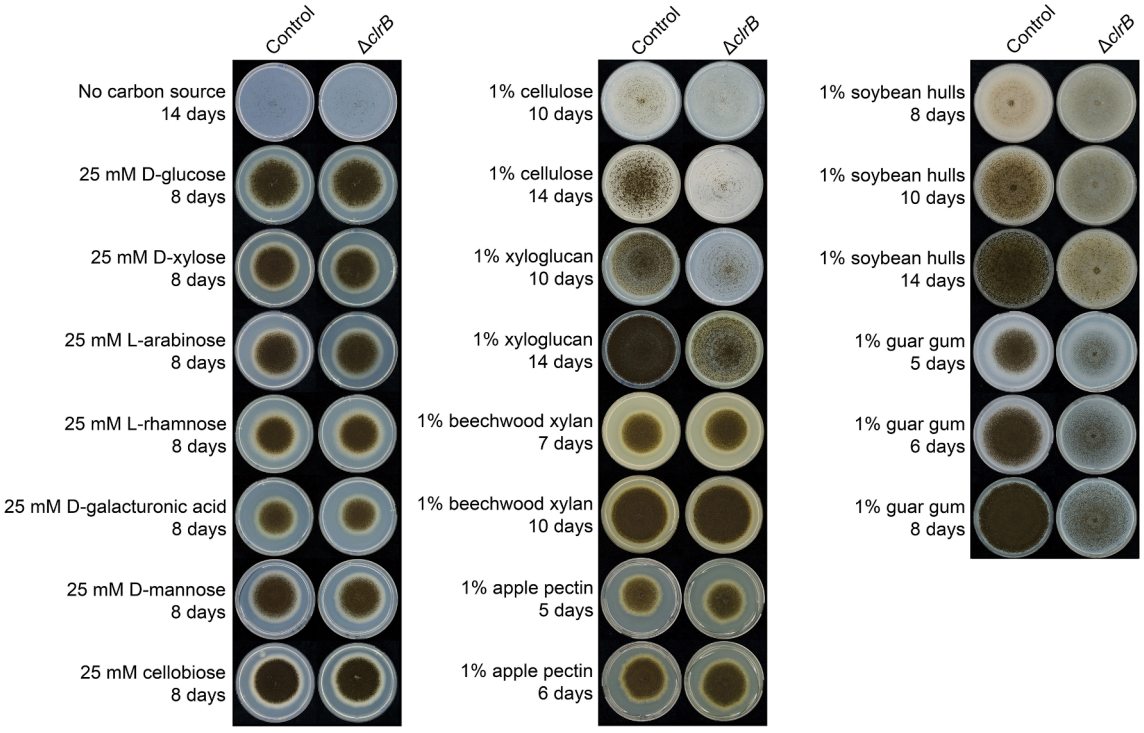


Fig. 1. Growth profile of the *A. niger* control (CBS 138852) and Δ*clrB* strain. The solid medium containing selected carbon sources was inoculated with 1000 spores and incubated at 30 °C for up to 14 days.

mutants to degrade galactomannan. This is supported by the observation that none of the XlnR-AraR-ClrA combinatorial deletion mutants showed substantial growth reduction on GG (**Figure S1**), which is almost exclusively composed of galactomannan.

3.2. Mannobiose is the inducer of ClrB in Aspergillus niger

Several studies have suggested that the inducer of CLR-2/ClrB/ManR is either cellobiose (Coradetti et al., 2013, 2012; Li et al., 2016; Ogawa et al., 2013, 2012) or mannobiose (Ogawa et al., 2013, 2012). Mannobiose-mediated activation of ClrB in *A. niger* has been hypothesized before (van Munster et al., 2020), but no studies have confirmed it so far.

To identify if either cellobiose or mannobiose is the inducer of ClrB in *A. niger*, the *A. niger* control (CBS 138852) and Δ*clrB* strain were cultivated in liquid medium containing either 2 mM cellobiose or 2 mM mannobiose as sole carbon source. RNA from mycelial samples was collected after 1 h incubation, and transcriptomic data were generated and analyzed. The expression level of *clrB* in the control strain was 3-fold higher in the mannobiose culture (147.1 FPKM) compared to the cellobiose culture (48.7 FPKM) (**Data S1A**). Analysis of CAZy, metabolic and sugar transporter genes indicated no downregulation (fold change < 0.5; padj < 0.01) in the Δ*clrB* mutant compared to the control when grown in medium containing 2 mM cellobiose (**Data S1A**). In contrast, in the 2 mM mannobiose cultures differential expression of 18 CAZy, two metabolic and three transporter genes was observed in the Δ*clrB* strain compared to the control (Table 1). The differentially expressed CAZy genes encode enzymes with a diverse specificity, including two xylanolytic genes (*axeA* and *gbgA*), six cellulolytic genes (*eglA*, *eglC, cbhB, cbhD, bgl4* and NRRL3_3383), six galactomannanolytic genes (*aglB, aglC, manA, mndA, mndB* and *haeA*), three pectinolytic genes (*abnA, rgaeA* and *rglA*) and one xyloglucanolytic gene (*xegA*) (Table 1). The downregulation of six cellulolytic and galactomannanolytic genes correlates with the expectation that ClrB is mainly involved in cellulose and mannan utilization. Although two metabolic genes (*larB* and *oahA*) were differentially expressed in the *clrB* deletion strain (Table 1), these results do not necessarily indicate an involvement of ClrB in the regulation of metabolic pathways. Of the three transporter genes which showed differential expression in the Δ*clrB* strain (Table 1), *ctA* showed the highest reduction in expression. Interestingly, the transporter encoded by this gene has previously been described as cellodextrin (including cellobiose) transporter (Lin et al., 2020). However, our data did not show the expression of this gene when grown on 2 mM cellobiose (**Data S1A**). This suggests that this gene may in fact encode a mannobiose transporter rather than a cellobiose transporter. This observation is further supported by a previous study, reporting that the CtA ortholog in *N. crassa*, CDT-1, showed competitive uptake of cellobiose and mannobiose, with preference for mannobiose (Hassan et al., 2019).

Overall, our data shows that mannobiose is most likely the inducer of ClrB in *A. niger*. However, a previous study reported that the overexpression of *A. niger clrB* could result in the production of some cellulases, but not the mannanase ManA, in the absence of an inducer (Gao et al., 2019). Furthermore, the expression of *clrB* is positively affected by the xylose-inducible TF XlnR (Raulo et al., 2016), which could likely explain the role of ClrB in the utilization of diverse substrates containing low levels, or even lacking mannobiose in their composition (Garrigues et al., 2022; Kun et al., 2021; Raulo et al., 2016).

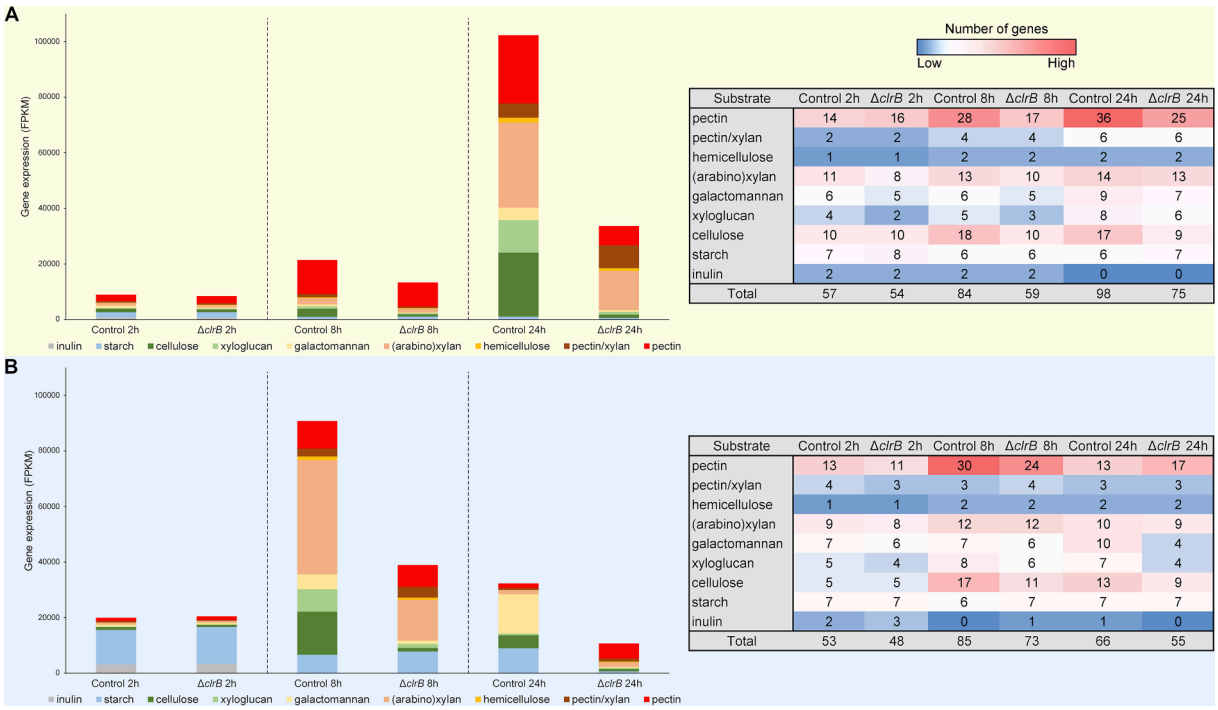
3.3. Transcriptome data shows slow activation of galactomannan degradation in soybean hulls and guar gum liquid cultures by Aspergillus niger

Transcriptome analysis of *A. niger* grown on SBH and GG was performed to study the phenotypic response of this fungus to the analyzed substrates at the molecular level. For this, pre-grown fungal mycelia were transferred to liquid medium containing 1 % GG or 1 % washed SBH. The expression of CAZyme-encoding genes was initially evaluated in the *A. niger* control (CBS 138852) strain to assess the major genes involved in the degradation of the tested substrates (Fig. 2).

On SBH, the overall expression of CAZyme-encoding genes was low after 2 h, showing an increase after 8 h (Fig. 2A). However, at 24 h, a relatively high increase in the expression of CAZy genes was observed,

Table 1
Differentially expressed genes in the Δ*clrB* strain compared to the control when cultivated in minimal medium containing 2 mM mannobiose as sole carbon source. Gene expression values represent FPKM values, while the fold change is based on Deseq2 calculation. Enzyme activity abbreviations are described in **Table S4**.

CAZyme-encoding genes								
Gene ID	**Gene name**	**Activity**	**CAZy family**	**Substrate**	**FPKM control**	**FPKM Δ*clrB***	**DeSeq2 fold change**	***padj***
NRRL3_3339	*axeA*	AXE	CE1	(Arabino)xylan	74.13	0.79	0.01	0.000
NRRL3_11773	*gbgA*	BXL	GH43 - CBM35	(Arabino)xylan	66.88	12.44	0.19	0.000
NRRL3_4917	*eglC*	EGL	GH5_5	Cellulose	147.06	0.06	0.00	0.000
NRRL3_10870	*cbhD*	CBH	GH6	Cellulose	24.28	0.24	0.01	0.000
NRRL3_3383	–	LPMO	AA9	Cellulose	26.67	1.20	0.05	0.000
NRRL3_8517	*bgl4*	BGL	GH1	Cellulose	1380.90	114.09	0.08	0.000
NRRL3_2584	*cbhB*	CBH	GH7 - CBM1	Cellulose	95.40	1.45	0.02	0.000
NRRL3_2585	*eglA*	EGL	GH5_5 - CBM1	Cellulose	219.80	0.26	0.00	0.000
NRRL3_5358	*aglB*	AGL	GH27	Galactomannan	1221.38	23.58	0.02	0.000
NRRL3_16	*aglC*	AGL	GH36	Galactomannan	93.25	41.27	0.45	0.000
NRRL3_8912	*manA*	MAN	GH5	Galactomannan	479.99	5.17	0.01	0.000
NRRL3_9612	*mndA*	MND	GH2	Galactomannan	1039.38	14.18	0.01	0.000
NRRL3_9051	*mndB*	MND	GH2	Galactomannan	2593.21	157.21	0.06	0.000
NRRL3_4916	*haeA*	AE	CE16	Galactomannan	280.84	0.87	0.00	0.000
NRRL3_92	*abnA*	ABN	GH43	Pectin	938.85	159.80	0.17	0.000
NRRL3_169	*rgaeA*	RGAE	CE12	Pectin	24.51	2.67	0.11	0.000
NRRL3_684	*rglA*	RGL	PL4_1	Pectin	97.06	5.87	0.06	0.000
NRRL3_1918	*xegA*	XG-EGL	GH12	Xyloglucan	25.14	10.21	0.42	0.000
Metabolic genes								
Gene ID	**Gene name**	**Activity**	**Metabolic pathway**		**FPKM control**	**FPKM Δ*clrB***	**DeSeq2 fold change**	***padj***
NRRL3_10868	*larB*	L-arabinose reductase	PCP		54.59	25.88	0.48	0.001
NRRL3_6354	*oahA*	oxaloacetate acetylhydrolase	TCA & glyoxylate cycles		3212.33	1439.89	0.46	0.000
Sugar transporter genes								
Gene ID	**Gene name**	**Specificity**			**FPKM control**	**FPKM Δ*clrB***	**DeSeq2 fold change**	***padj***
NRRL3_10052	*xltC*	major facilitator superfamily glucose transporter			176.29	37.67	0.22	0.000
NRRL3_3028	*ctA*	major facilitator superfamily, sugar/inositol transporter-like protein			1657.42	14.81	0.01	0.000
NRRL3_5614	–	major facilitator superfamily, sugar/inositol transporter-like protein			206.15	55.66	0.28	0.000



Substrate	Control 2h	ΔclrB 2h	Control 8h	ΔclrB 8h	Control 24h	ΔclrB 24h
pectin	14	16	28	17	36	25
pectin/xylan	2	2	4	4	6	6
hemicellulose	1	1	2	2	2	2
(arabino)xylan	11	8	13	10	14	13
galactomannan	6	5	6	5	9	7
xyloglucan	4	2	5	3	8	6
cellulose	10	10	18	10	17	9
starch	7	8	6	6	6	7
inulin	2	2	2	2	0	0
Total	57	54	84	59	98	75

Substrate	Control 2h	ΔclrB 2h	Control 8h	ΔclrB 8h	Control 24h	ΔclrB 24h
pectin	13	11	30	24	13	17
pectin/xylan	4	3	3	4	3	3
hemicellulose	1	1	2	2	2	2
(arabino)xylan	9	8	12	12	10	9
galactomannan	7	6	7	6	10	4
xyloglucan	5	4	8	6	7	4
cellulose	5	5	17	11	13	9
starch	7	7	6	7	7	7
inulin	2	3	0	1	1	0
Total	53	48	85	73	66	55

Fig. 2. Schematic representation of the relative expression of CAZyme-encoding genes in *A. niger* control (CBS 138852) and Δ*clrB* strains grown on soybean hulls (A) or guar gum (B). Graphs illustrate the cumulative expression of genes associated with the degradation of major plant biomass components (indicated by different colors). Tables describe the number of genes associated with the degradation of each substrate with a FPKM > 20.

indicating a slow adaptation of *A. niger* to the degradation of SBH. This is in contrast with the gene expression pattern observed on another dicot, sugar beet pulp, where the overall expression of CAZy genes was only slightly higher at 24 h compared to 8 h (Garrigues et al., 2022). The total number of CAZy genes showing an expression of FPKM > 20 also increased over time, with 57, 84 and 98 CAZy genes in total after 2, 8 and 24 h, respectively (Fig. 2A).

At the initial stage of growth, the α-glucosidase encoding gene *agdB* showed the highest expression in the control strain (**Data S1B**). The high expression of *agdB*, as well as additional amylolytic genes (e.g., *glaA*, *amyA* and *agdA*) indicated the utilization of starch after 2 h on SBH. However, the starch content of SBH used in our study is only 0.2 % (data not shown), which is in line with previous studies reporting<1 % starch in SBH (Karr-Lilienthal et al., 2005; Rodiek and Stull, 2007). None of the genes related to the degradation of other SBH components were expressed at relatively high levels, suggesting that *A. niger* did not degrade other polymers at this stage of growth (Fig. 2A).

In contrast, after 8 h, the expression of three *endo*-arabinanase encoding genes (*abnA, abnC* and NRRL3_3855) was the highest (Fig. 3 and **Data S1B**). In accordance with the highly expressed *endo*-arabinanase encoding genes, additional pectinolytic genes were most abundantly expressed, accounting for a total number of 28 at this time point (Fig. 2A). In general, (hemi-)cellulolytic genes showed the highest expression levels only after 24 h, including genes encoding cellobiohydrolases (*cbhA, cbhD*), endoxylanases (*xlnA*, *xlnC/xynA*), arabinoxylan arabinofuranohydrolase (*axhA*), acetyl xylan esterase (*axeA*), hemicellulose acetyl esterase (*haeA*) and xyloglucanases (*xegA*, *eglA*) (Fig. 3 and **Data S1B**).

In contrast to SBH, *A. niger* grown in GG cultures showed the highest expression of CAZyme-encoding genes after 8 h, with a substantial decrease in expression after 24 h (Fig. 2B). The expression level of *clrB* was also the highest in the control strain grown on SBH for 24 h and GG for 8 h (Fig. 4 and **Data S1C**), correlating with the expression profile of CAZyme-encoding genes. This correlation supports the major role that ClrB plays in the degradation of both substrates.

At the initial stages of growth on GG, several amylolytic genes (*glaA*, *aamA*, *agdA*, *agdB*) were highly expressed (Fig. 2B and **Data S1B**). Moreover, the endoinulinase encoding gene *inuE* and invertase encoding gene *sucA* were also highly expressed after 2 h on GG. Even though starch and inulin are not present in GG, it was previously shown that GG contains trace amounts of various components that might induce the expression of a broad set of CAZyme-encoding genes, including amylases and/or inulinases in *A. niger* (Coconi Linares et al., 2019). Additionally, a trace component-mediated induction of amylolytic and inulinolytic gene expression in the 2 h GG condition compared to the 2 h SBH condition might be explained by the fact that the SBH used in this study was previously washed, while GG was not. The washing procedure removes a large proportion of (free) monosaccharides or short oligosaccharides from the substrate composition, decreasing the availability of putative inducer molecules.

After 8 h a large number of pectinolytic genes were expressed in GG, but these were not among the genes showing the highest expression levels (Fig. 2B and **Data S1B**). Several genes that showed high expression values in the SBH culture after 24 h were also highly expressed in GG at the 8 h time point. These include *axeA*, *xlnA*, *xlnC/xynA, axhA*, *xegA* and *haeA* (Fig. 3 and **Data S1B**). Additionally, the endoglucanase encoding gene NRRL3_4917 and the cellobiohydrolase encoding gene *cbhB* showed high expression levels, indicating a strong response to the presence of low amounts of cellulose. The expression data indicated that the GG cultures showed the strongest genetic response to the presence of its main constituent, galactomannan, only after 24 h, showing the highest expression of the α-galactosidase encoding *aglB*, the hemicellulose acetyl esterase encoding *haeA*, the β-1,4-endomannanase encoding *manA* and the two β-1,4-mannosidase encoding genes *mndA* and *mndB* (Fig. 3 and **Data S1B**). Comparison of the expression profile of galactomannan-specific genes in the GG and SBH conditions at each time point suggests that the degradation of galactomannan mainly occurs at a later stage of growth, possibly even after 24 h of growth in case of SBH.

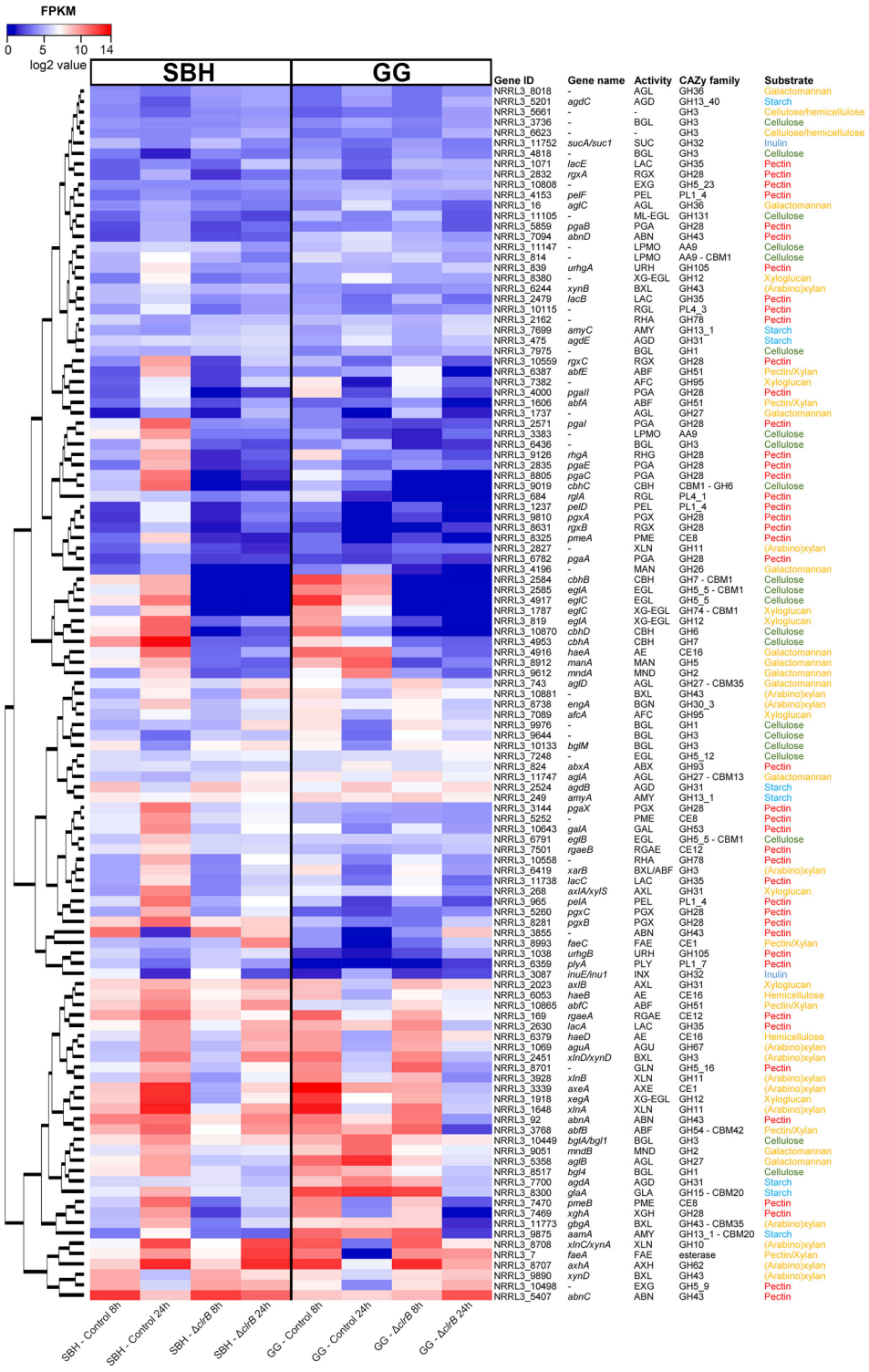


Fig. 3. Hierarchical clustering of CAZyme-encoding genes in *A. niger* control (CBS 138852) and Δ*clrB* strains. Data originated from 8 and 24 h of culturing in 1 % soybean hulls (SBH) or 1 % guar gum (GG) liquid media. The substrates associated with the corresponding genes are indicated by different colors. Enzyme activity abbreviations are described in Table S4.

3.4. Transcriptomic analysis of the clrB deletion mutant confirms the key role of ClrB in the regulation of key cellulases, galactomannanases and xyloglucanases in Aspergillus niger

To evaluate the role of ClrB in the regulation of CAZymes involved in the degradation of SBH and GG, we compared the transcriptome of the Δ*clrB* mutant to that of the control described above (Fig. 3 and **Data S1B**). CAZyme-encoding genes were evaluated based on substrate specificity for both growth conditions (SBH and GG) during the experimental time course. Only seven genes showed downregulation in the Δ*clrB* mutant compared to the control on SBH after 2 h (Fig. 5 and **Table S3**). However, the number of downregulated genes increased to 33 and 78 after 8 h and 24 h on SBH, respectively. Interestingly, all the genes which were downregulated in the *clrB* deletion mutant at 2 or 8 h

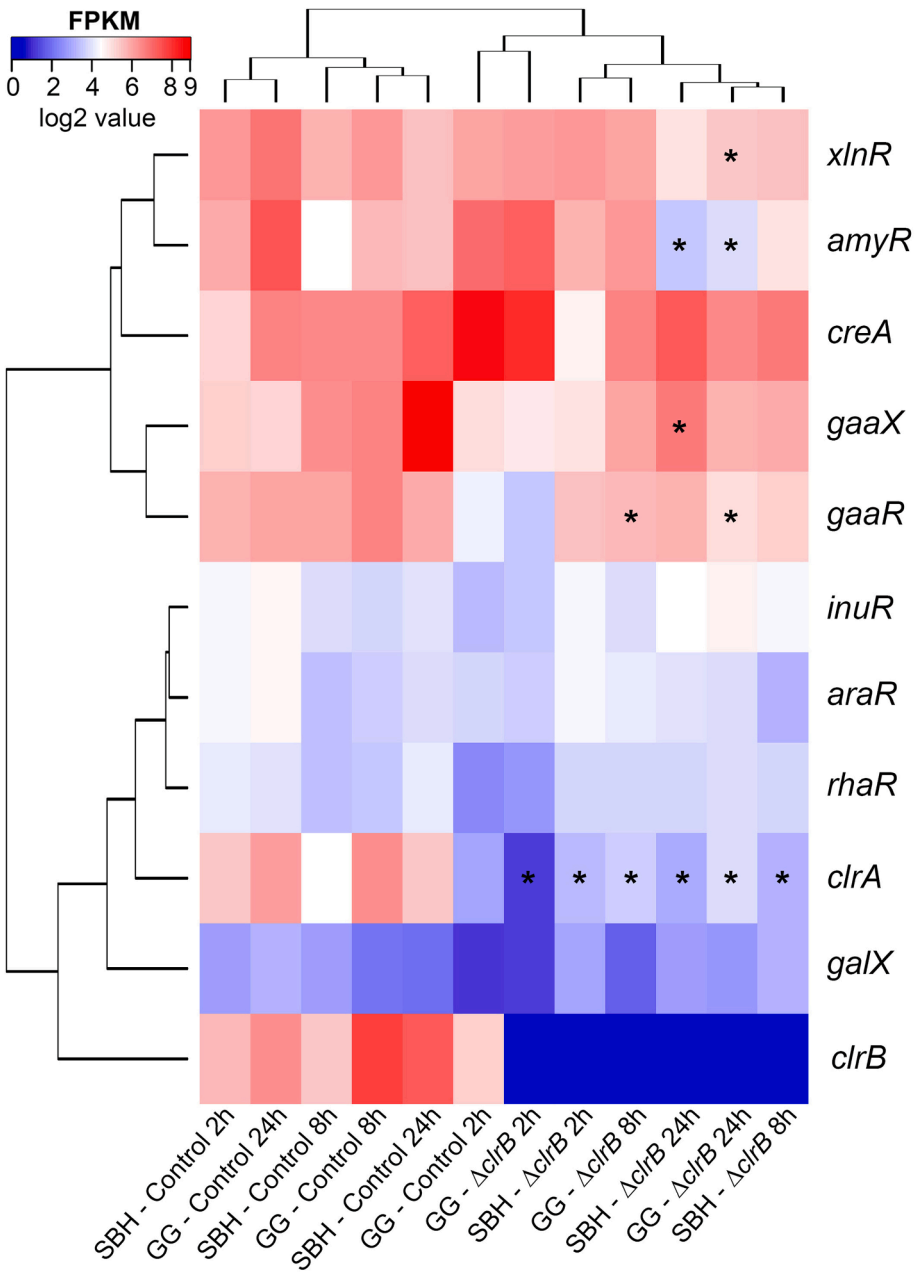


Fig. 4. Hierarchical clustering of transcription factor genes in *A. niger* control (CBS 138852) and Δ*clrB* strains. Data originated from 2, 8 and 24 h of culturing in 1 % soybean hulls (SBH) or 1 % guar gum (GG) liquid media. Downregulated genes (fold change < 0.5; padj < 0.01) in Δ*clrB* compared to the control are indicated by an asterisk (*). The analyzed genes include the genes encoding the carbon catabolite repressor CreA, the (hemi-)cellulolytic regulators ClrA and ClrB, the xylanolytic regulator XlnR, the arabinanolytic regulator AraR, the amylolytic regulator AmyR, the inulinolytic regulator InuR, the regulator of L-rhamnose utilization RhaR, the regulator of D-galactose utilization GalX and the activator and repressor of D-galacturonic acid utilization GaaR and GaaX, respectively.

were also downregulated at 24 h, so no unique downregulated genes were found in this mutant at early time points (Fig. 5).

Compared to the SBH cultures, a lower number of genes were affected by the deletion of *clrB* on GG. This includes 6, 30 and 36 downregulated genes after 2, 8 and 24 h, respectively. Thirteen of the downregulated genes were unique to the 8 h time point, while 19 were unique to the 24 h time point in the GG culture (Fig. 5 and **Table S3**). In total, 80 CAZyme-encoding genes showed downregulation in Δ*clrB* on either SBH or GG (**Table S3**). Of these, 45 genes were downregulated on SBH and GG at identical time points (Fig. 5).

After 2 h, the deletion of *clrB* showed low impact, downregulating only *bgl4*, *aglB*, *mndA*, *rgaeA* and *eglA*. In contrast, the 8 h time point showed the downregulation of 24 genes on both substrates, notably including eight genes involved in cellulose degradation (Fig. 5). A similar set of eight cellulase genes was downregulated at the 24 h time point, of which *bgl4*, *cbhA*, *cbhB*, *eglA* and *eglC* were downregulated at both 8 and 24 h time points (Fig. 5). Previously, it was shown that XlnR plays a major role in the regulation of cellulases in *A. niger* by controlling the expression of both *clrA* and *clrB* (Raulo et al., 2016). However, our study shows that the expression of *clrA* is (partially) dependent on ClrB in *A. niger* (Fig. 4). This indicates that the decreased expression of *clrA* in a Δ*xlnR* mutant (Raulo et al., 2016) might also be indirectly mediated through ClrB. Moreover, the reduced expression of *clrA* can further facilitate the reduction of cellulase gene expression in *A. niger*.

Besides cellulase genes, three galactomannanolytic genes (*aglB*, *manA* and *haeA*) were downregulated after 8 h of growth on SBH and GG (Fig. 5). These genes seem to be activated by ClrB, even when the general response of *A. niger* to galactomannan degradation is low in case of SBH (Fig. 2A). However, the 24 h time point showed the downregulation of additional galactomannanolytic genes, including *aglC*, NRRL3_4196 (putative endomannanase), *mndA* and *mndB* (Fig. 5). The control of *manA* by ClrB in *A. niger* was shown in a previous study where a constitutively active version of ClrB resulted in elevated levels of the β-mannanase ManA, but no data was provided about the production of additional major mannanolytic enzymes by this mutant (Gao et al., 2019). Regulation of *manC* and *mndB* by *N. crassa* CLR-2 and *A. nidulans*

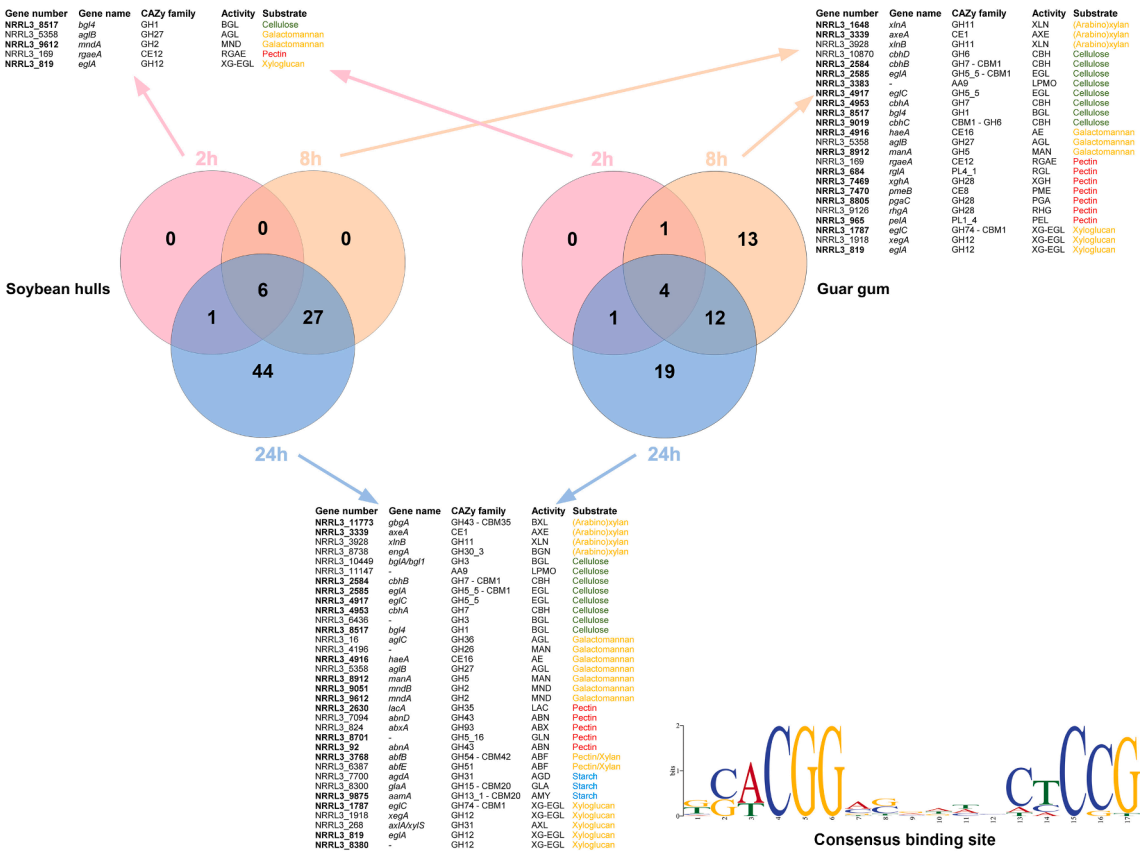


Fig. 5. Downregulated CAZyme-encoding genes in *A. niger* grown on 1 % soybean hulls or 1 % guar gum. Venn-diagrams indicate the number of downregulated (fold change < 0.5; padj < 0.01) genes in Δ*clrB* strain compared to the control at different time points. Only the genes which are downregulated in both growth conditions are listed for each time point. The substrates associated with the corresponding genes are indicated by different colors. Arrow colors match those of the different time points. Bold gene numbers indicate the genes which possess the ClrB consensus binding site [5′-CGGN$_8$CCG-3′] in their promoter region. Enzyme activity abbreviations are described in **Table S4**.

ClrB has been reported before (Coradetti et al., 2013). However, neither of these orthologs have been shown to control the expression of genes encoding α-galactosidases or acetyl mannan esterases, which are accessory enzymes involved in galactomannan utilization. In contrast, *A. oryzae* ManR was shown to control the expression of a GH27 and a GH36 α-galactosidase gene (*aglB* and *aglC*, respectively), as well as the Carbohydrate Esterase Family 16 (CE16) acetyl mannan esterase encoding gene *ameA* (Ogawa et al., 2013, 2012). Our data indicates that the ClrB-mediated activation of galactomannanases in *A. niger* is similar to that of the ManR in *A. oryzae*, indicating a divergent regulation of mannan utilization compared to *N. crassa* or *A. nidulans*.

Only a few pectinolytic genes were downregulated in the Δ*clrB* mutant after 8 h or 24 h on SBH and GG (seven and five, respectively) (Fig. 5), and none of these genes showed overlap between the two time points, indicating that they might not be under direct control of ClrB.

Previous studies showed that CLR-2/ClrB orthologs can affect the expression of xylanolytic genes as well as overall xylanase activity in *N. crassa* and *A. nidulans* when grown on Avicel (Coradetti et al., 2013, 2012). However, this was not the case for *A. oryzae* ManR, which showed no significant effect on xylanolytic gene expression on Avicel (Ogawa et al., 2013). Although our growth condition was different, *A. niger* Δ*clrB* did not show extensive involvement of ClrB in the regulation of (arabino)xylan utilization. Likewise, it was shown that *P. oxalicum* ClrB (Li et al., 2015) and *T. cellulolyticus* TclB2 (Fujii et al., 2021) are not strictly required for xylanolytic gene expression. Only some genes involved in (arabino)xylan degradation were downregulated on both SBH and GG after 8 h and 24 h (three and four, respectively), two of which (*axeA* and *xlnB*) were downregulated at both time points (Fig. 5). A previous study showed that the expression of *xlnB* (*xynA*) decreased in *A. niger* Δ*clrB* on wheat straw (Raulo et al., 2016). The minor involvement of *A. niger* ClrB in the process of (arabino)xylan utilization was supported by the unaffected growth of Δ*clrB* mutant on xylan (Fig. 1), as well as by the results of our previous study, where an *A. niger* Δ*clrB* mutant showed an unexpected growth improvement on an (arabino)xylan-rich substrate, wheat bran (Kun et al., 2021). Those results suggest the existence of an unknown interaction between XlnR and ClrB, where the presence of ClrB negatively affects the production of xylanolytic enzymes while XlnR is the dominant transcription factor in the degradation of the substrate (Kun et al., 2021).

The expression of xyloglucanolytic genes was also affected in Δ*clrB* (Fig. 5). Reduced xyloglucanolytic activities are also indicated by the reduced growth of Δ*clrB* on xyloglucan (Fig. 1). The importance of CLR-2 in the degradation of xyloglucan was reported before in *N. crassa* (Samal et al., 2017), but no evidence has been given for involvement of ClrB/ManR orthologs in xyloglucan degradation in *P. oxalicum*, *A. nidulans* or *A. oryzae*. To this point, it is not fully known to which extent the regulation of xyloglucan degradation by ClrB is conserved in filamentous fungi.

The set of 45 genes identified in this study that were simultaneously downregulated in Δ*clrB* on SBH and GG (**Table S3**) was used to *in silico* predict the consensus binding site of ClrB in *A. niger* (Fig. 5). Our predicted consensus binding site [5′-CGGN$_8$CCG-3′] matched the one previously described for *A. nidulans* ClrB (Li et al., 2016) and shows high similarity to the experimentally determined [5′-CGGN$_{11}$CCG-3′] binding site in *N. crassa* (Craig et al., 2015).

Considering each set of downregulated genes per different growth conditions and time points, only three genes were consistently downregulated in all conditions, *bgl4*, *aglB* and *eglA*, indicating the direct ClrB-

mediated activation of their expression in *A. niger* at all time points. Interestingly, binding site analysis did not indicate the presence of a [5′-CGGN$_8$CCG-3′] site in the promoter region of *aglB*. However, a [5′-CGN$_9$CCG-3′] motif could be identified within the 1000 bp promoter region of *aglB*, indicating that the binding site of ClrB in *A. niger* might be slightly different from the sequence reported for *A. nidulans* ClrB (Li et al., 2016), or that slightly different sequence motifs to the one predicted in this study may be also recognized by this activator. Nevertheless, the identification of the binding site in *A. niger* requires experimental validation.

3.5. ClrB controls the expression of several sugar transporter genes, but shows no direct control of sugar metabolic pathways in Aspergillus niger

The expression of putative sugar transporter and metabolic genes was assessed on both SBH and GG cultures at all time points (2, 8 and 24 h) to evaluate the effect of the deletion of *clrB* on the overall utilization of these substrates. Only the putative cellodextrin transporter gene, *ctA* (Lin et al., 2020), showed consistent downregulation at all time points in both growth conditions (Table 2 and **Data S1D**). The promoter region of *ctA* contains the consensus binding site of ClrB (5′-CGGN$_8$CCG-3′), likely indicating direct control by ClrB. A Cdt-1 cellodextrin transporter, the putative ortholog of *A. niger* CtA, was also identified within the conserved regulon of CLR-2/ClrB in *N. crassa* and *A. nidulans* (Coradetti et al., 2013). Additionally, the putative transporter encoding NRRL3_5614 was downregulated after 8 h and 24 h on both substrates, while NRRL3_8663 showed downregulation only on SBH after 8 and 24 h in Δ*clrB* (Table 2 and **Data S1D**). However, NRRL3_8663 was upregulated in Δ*clrB* on GG after 8 h, similar to NRRL3_10866, which showed no differential expression when grown on SBH. NRRL3_8663 also showed increased expression levels in the control strain on SBH compared to GG (Table 2 and **Data S1D**). Based on the higher L-arabinose content of SBH determined by the sugar composition analysis of both substrates (**Table S2**), this data correlates with previous observations suggesting the putative involvement of NRRL3_8663 in arabinose transport (Peng et al., 2018). In contrast, no function has been associated yet with NRRL3_5614 and NRRL3_10866 genes (Peng et al., 2018). While only a low number of transporter genes showed differential expression after 2 or 8 h of growth, several additional transporter genes were differentially expressed on SBH and GG after 24 h in Δ*clrB*. This includes 10 upregulated and 14 downregulated genes in SBH and 12 upregulated and 11 downregulated genes in GG. Only seven genes were upregulated, and seven genes were downregulated in both conditions (Table 2 and **Data S1D**). Considering the role of ClrB as a transcriptional activator, the large number of upregulated genes after 24 h of growth indicates that several transporter genes are differentially expressed most likely due to an indirect effect triggered by the deletion of *clrB*.

Only a low number of metabolic genes showed differential expression in Δ*clrB* grown on SBH or GG for 2 or 8 h. The affected genes belong to several sugar metabolic pathways (**Data S1E**), indicating no substantial involvement of ClrB in the regulation of any major sugar metabolic pathway. In contrast, a large number of metabolic genes were downregulated on both substrates in Δ*clrB* after 24 h of growth (**Data S1E**). Similar to the relatively large number of differentially expressed transporter genes after 24 h of growth, the differential expression of these metabolic genes is most likely a result of an indirect effect caused by the deletion of *clrB*. Moreover, the unaffected growth of Δ*clrB* on the major constituent monosaccharides (Fig. 1) further supports the lack of involvement of ClrB in the regulation of major metabolic pathways.

4. Conclusions

In conclusion, we show that ClrB plays a major role in the regulation of genes necessary for the utilization of SBH and GG in *A. niger* (Fig. 6). ClrB is essential to maintain growth on cellulose and galacto(mannan) and is highly involved in the degradation of xyloglucan. Gene expression

Table 2
Downregulated sugar transporter genes in the *A. niger* Δ*clrB* strain compared to the control (CBS 138852). Strains were cultivated on soybean hulls or guar gum for 2, 8 and 24 h. Gene expression values represent FPKM values. Gene downregulation is indicated by bold and underlined expression values. Associated fold-change and *p*adj values are described in **Data S1D**.

Gene ID	Gene name	Classification (based on (Peng et al., 2018))	Soybean hulls						Guar gum					
			Control 2 h	Control 8 h	Control 24 h	Δ*clrB* 2 h	Δ*clrB* 8 h	Δ*clrB* 24 h	Control 2 h	Control 8 h	Control 24 h	Δ*clrB* 2 h	Δ*clrB* 8 h	Δ*clrB* 24 h
NRRL3_3028	*ctA*	cellodextrin or lactose ST*	713.60	597.11	1812.91	**235.02**	**103.49**	**67.18**	40.59	2254.97	3167.45	**11.02**	**127.91**	**165.11**
NRRL3_5614	–	unknown ST	93.69	168.55	336.83	62.41	**77.69**	**152.49**	9.61	244.93	237.57	7.05	**89.08**	**104.46**
NRRL3_8663	–	glucose or pentose ST	4.70	23.29	543.34	4.76	**11.68**	**258.95**	25.79	16.38	9.42	25.44	68.08	1.88
NRRL3_958	*gatA*	D-galacturonic or quinic acid ST	50.93	60.84	1539.72	36.88	50.63	**86.70**	27.36	50.10	35.69	25.22	35.12	20.57
NRRL3_10052	*xltC*	glucose or pentose ST	12.73	13.59	259.83	5.39	11.77	**18.02**	88.47	17.25	391.31	63.84	10.44	**9.17**
NRRL3_2828	–	pentose or glycerol ST	2.98	8.64	62.36	2.23	1.61	**4.44**	1.28	14.66	10.17	1.70	10.30	24.00
NRRL3_1652	–	xylose ST	237.92	65.90	402.13	260.42	88.08	**51.05**	434.71	616.68	1209.57	392.31	763.31	**309.64**
NRRL3_4569	–	pentose or glycerol ST	18.02	14.48	123.39	14.25	19.00	**24.56**	56.52	26.76	176.85	42.58	35.27	**25.51**
NRRL3_235	–	pentose or glycerol ST	6.43	3.76	44.34	3.54	5.13	**11.09**	16.57	22.93	58.10	12.66	20.12	**12.43**
NRRL3_11054	–	unknown ST	533.06	595.07	1456.22	476.72	505.20	**397.73**	31.03	193.66	113.51	28.98	127.69	187.28
NRRL3_3702	–	inositol or fructose ST	7.40	7.98	11.65	7.59	11.01	3.19	23.86	41.21	24.14	19.47	39.39	**5.81**
NRRL3_11036	–	D-galacturonic or quinic acid ST	20.19	7.29	21.10	20.37	6.30	**8.05**	5.06	0.85	9.68	4.52	1.37	10.19
NRRL3_3594	–	maltose or sucrose ST	58.62	34.18	35.62	71.44	42.65	**17.02**	60.93	43.36	21.76	71.69	58.55	16.32
NRRL3_11715	*xltA*	xylose ST	6.63	17.28	874.82	4.94	15.75	**441.07**	49.21	951.11	580.11	48.93	626.04	**31.87**
NRRL3_728	–	cellodextrin or lactose ST	34.47	69.82	175.25	31.42	56.10	**101.13**	3.89	149.49	12.30	3.93	102.16	68.02
NRRL3_8653	–	maltose or sucrose ST	72.31	24.83	23.79	69.30	20.51	19.90	3.05	29.50	48.55	2.99	32.91	**20.72**
NRRL3_11807	–	inositol or fructose ST	303.85	278.10	89.24	232.38	227.20	84.06	624.96	132.60	427.84	716.42	108.82	**76.52**
NRRL3_3879	*mstH*	hexose ST	263.35	79.14	48.78	199.68	81.33	55.19	1194.03	129.03	1591.99	1104.57	116.62	**9.22**

* ST = sugar transporter.

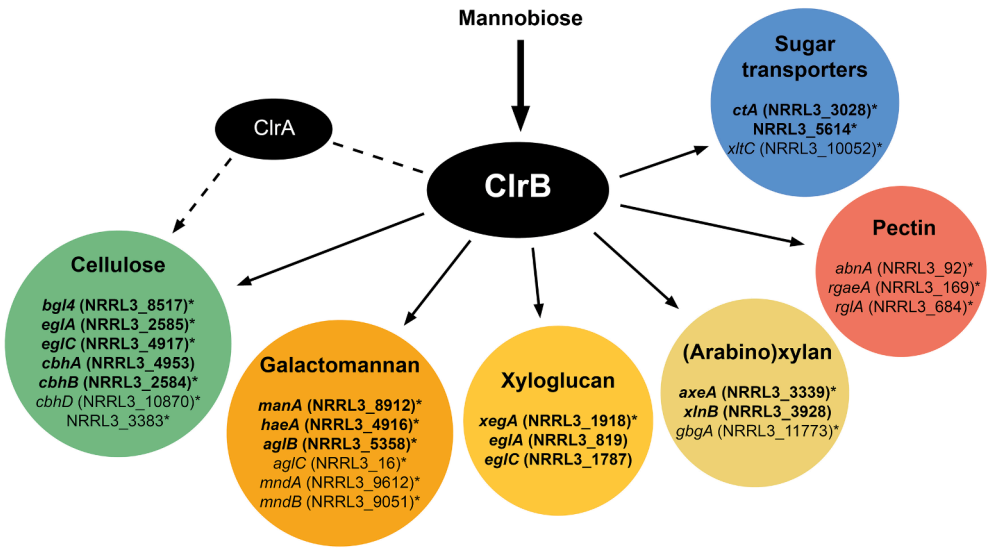


Fig. 6. The role of ClrB in *A. niger*. Schematic presentation of the major differentially expressed CAZyme- or sugar transporter-encoding genes in the *A. niger* Δ*clrB* strain compared to the control strain (CBS 138852). Genes which were consistently downregulated in the *A. niger* Δ*clrB* strain after 8 or 24 h of growth in 1 % soybean hulls or 1 % guar gum liquid cultures are highlighted in bold. Genes which were downregulated in the 2 mM mannobiose liquid culture are indicated by an asterisk (*).

data also shows that ClrB regulates (arabino)xylanolytic genes, although to a lesser extent. Moreover, the role of ClrB extends beyond (hemi-) cellulose degradation through the partial (in)direct regulation of several pectinolytic genes (e.g., *rglA* or *abnA*) (Fig. 6). CLR-2/ClrB/ManR orthologs have been studied in *N. crassa*, *A. nidulans* (Coradetti et al., 2012), *A. oryzae* (Ogawa et al., 2012) and *P. oxalicum* (Li et al., 2015). Our data suggests both conserved and divergent functions of the *A. niger* ClrB compared to its orthologs across these organisms, even within the genus *Aspergillus*. The involvement of CLR-2/ClrB/ManR orthologs in the regulation of cellulose utilization appears to be well conserved in the studied species. However, growth on the cellulose constituent, cellobiose, was not affected by the deletion of *clrB* in *A. niger*, while a strong growth reduction on cellobiose was observed for an *A. nidulans clrB* deletion strain (Coradetti et al., 2012). Additionally, the regulation of mannanolytic genes in *A. niger* differs from that of *A. nidulans*, where the regulation of major mannanolytic genes has been associated with the AN6832 paralog of ClrB (Li et al., 2016), which is absent in *A. niger* (Kunitake and Kobayashi, 2017). In contrast, the subset of mannanolytic genes regulated by the *A. niger* ClrB is similar to the ones regulated by ManR in *A. oryzae*, including several accessory enzymes, such as α-galactosidases or acetyl mannan esterases (Ogawa et al., 2013, 2012). Additionally, *A. niger* ClrB is involved in the regulation of xyloglucanolytic activities, which has been suggested in *N. crassa* as well (Samal et al., 2017), however, no evidence has been shown about this role in other species. Gene expression data in our study also indicates the involvement of ClrB in the regulation of several sugar transporter genes, including *ctA* (Fig. 6), but shows no (direct) involvement in the regulation of primary metabolic pathways.

Finally, we show that mannobiose is most likely the inducer of ClrB, and that the expression of the cellulolytic regulator gene *clrA* is (partially) dependent on ClrB in *A. niger* (Fig. 6). These results highlight substantial differences between the signaling pathways in *A. niger* compared to *N. crassa*, where a cellobiose-activated CLR-1 transcription factor is necessary to achieve increased expression levels of *clr-2* (Coradetti et al., 2012). The determination of the key genes regulated by ClrB, as well as its inducing molecule in *A. niger*, allows us to improve the production of enzymes required for the degradation of plant biomass substrates rich in galactomannan, cellulose and/or xyloglucan through targeted gene editing and the optimization of culture conditions.

CRediT authorship contribution statement

Roland S. Kun: Investigation, Formal analysis, Visualization, Writing – original draft. **Sandra Garrigues:** Supervision, Writing – review & editing. **Mao Peng:** Formal analysis. **Keykhosrow Keymanesh:** Investigation, Formal analysis. **Anna Lipzen:** Data curation, Formal analysis. **Vivian Ng:** Project administration. **Sravanthi Tejomurthula:** Investigation, Formal analysis. **Igor V. Grigoriev:** Supervision. **Ronald P. de Vries:** Conceptualization, Resources, Funding acquisition, Supervision, Writing – review & editing.

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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Appendix A. Supplementary material

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References

Alazi, E., Niu, J., Kowalczyk, J.E., Peng, M., Aguilar Pontes, M.V., van Kan, J.A.L., Visser, J., de Vries, R.P., Ram, A.F.J., 2016. The transcriptional activator GaaR of *Aspergillus niger* is required for release and utilization of D-galacturonic acid from pectin. FEBS Lett. 590, 1804–1815. https://doi.org/10.1002/1873-3468.12211.

Aspinall, G.O., Hunt, K., Morrison, I.M., 1966. Polysaccharides of soy-beans. Part II. Fractionation of hull cell-wall polysaccharides and the structure of a xylan. J. Chem. Soc. C Org. 21, 1945–1949. https://doi.org/10.1039/j39660001945.

Bailey, T.L., Elkan, C., 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc. Int. Conf. Intell. Syst. Mol. Biol. 2, 28–36.

Battaglia, E., Visser, L., Nijssen, A., van Veluw, G.J., Wösten, H.A.B., de Vries, R.P., 2011. Analysis of regulation of pentose utilisation in *Aspergillus niger* reveals evolutionary adaptations in Eurotiales. Stud. Mycol. 69, 31–38. https://doi.org/10.3114/sim.2011.69.03.

Beier, S., Hinterdobler, W., Bazafkan, H., Schillinger, L., Schmoll, M., 2020. CLR1 and CLR2 are light dependent regulators of xylanase and pectinase genes in *Trichoderma reesei*. Fungal Genet. Biol. 136, 103315 https://doi.org/10.1016/J.FGB.2019.103315.

Benocci, T., Aguilar-Pontes, M.V., Zhou, M., Seiboth, B., de Vries, R.P., 2017. Regulators of plant biomass degradation in ascomycetous fungi. Biotechnol. Biofuels 10, 152. https://doi.org/10.1186/s13068-017-0841-x.

Coconi Linares, N., Di Falco, M., Benoit-Gelber, I., Gruben, B.S., Peng, M., Tsang, A., Mäkelä, M.R., de Vries, R.P., 2019. The presence of trace components significantly broadens the molecular response of *Aspergillus niger* to guar gum. N. Biotechnol. 51, 57–66. https://doi.org/10.1016/j.nbt.2019.02.005.

Coradetti, S.T., Craig, J.P., Xiong, Y., Shock, T., Tian, C., Glass, N.L., 2012. Conserved and essential transcription factors for cellulase gene expression in ascomycete fungi. Proc. Natl. Acad. Sci. U. S. A. 109, 7397–7402. https://doi.org/10.1073/pnas.1200785109.

Coradetti, S.T., Xiong, Y., Glass, N.L., 2013. Analysis of a conserved cellulase transcriptional regulator reveals inducer-independent production of cellulolytic enzymes in *Neurospora crassa*. Microbiologyopen 2, 595–609. https://doi.org/10.1002/mbo3.94.

Craig, J.P., Coradetti, S.T., Starr, T.L., Glass, N.L., 2015. Direct target network of the *Neurospora crassa* plant cell wall deconstruction regulators CLR-1, CLR-2, and XLR-1. MBio 6, e01452–e01515. https://doi.org/10.1128/mBio.01452-15.

de Vries, R.P., Visser, J., 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol. Mol. Biol. Rev. 65, 497–522. https://doi.org/10.1128/mmbr.65.4.497-522.2001.

de Vries, R.P., van den Broeck, H.C., Dekkers, E., Manzanares, P., de Graaff, L.H., Visser, J., 1999. Differential expression of three α-galactosidase genes and a single β-galactosidase gene from *Aspergillus niger*. Appl. Environ. Microbiol. 65, 2453–2460. https://doi.org/10.1128/aem.65.6.2453-2460.1999.

de Vries, R.P., Burgers, K., van de Vondervoort, P., Frisvad, J., Samson, R., Visser, J., 2004. A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production. Appl. Environ. Microbiol. 70, 3954–3959. https://doi.org/10.1128/AEM.70.7.3954-3959.2004.

de Vries, R.P., Patyshakuliyeva, A., Garrigues, S., Agarwal-Jans, S., 2020. The current biotechnological status and potential of plant and algal biomass degrading/modifying enzymes from Ascomycete Fungi. In: Nevalainen, H. (Ed.), Grand Challenges in Fungal Biotechnology. Springer International Publishing, Cham, pp. 81–120. https://doi.org/10.1007/978-3-030-29541-7_4.

Fujii, T., Inoue, H., Matsushika, A., 2021. The transcription factor gene *tclB2* regulates mannanolytic enzyme production in the fungus *Talaromyces cellulolyticus*. Appl. Biochem. Biotechnol. 193, 3163–3172. https://doi.org/10.1007/S12010-021-03588-8.

Gao, L., Xu, Y., Song, X., Li, S., Xia, C., Xu, J., Qin, Y., Liu, G., Qu, Y., 2019. Deletion of the middle region of the transcription factor ClrB in *Penicillium oxalicum* enables cellulase production in the presence of glucose. J. Biol. Chem. 294, 18658–18697. https://doi.org/10.1074/JBC.RA119.010863.

Garrigues, S., Kun, R.S., Peng, M., Bauer, D., Keymanesh, K., Lipzen, A., Ng, V., Grigoriev, I.V., de Vries, R.P., 2022. Unraveling the regulation of sugar beet pulp utilization in the industrially relevant fungus *Aspergillus niger*. iScience 25, 104065. https://doi.org/10.1016/J.ISCI.2022.104065.

Häkkinen, M., Valkonen, M.J., Westerholm-Parvinen, A., Aro, N., Arvas, M., Vitikainen, M., Penttilä, M., Saloheimo, M., Pakula, T.M., 2014. Screening of candidate regulators for cellulase and hemicellulase production in *Trichoderma reesei* and identification of a factor essential for cellulase production. Biotechnol. Biofuels 7, 14. https://doi.org/10.1186/1754-6834-7-14.

Hasper, A.A., Dekkers, E., van Mil, M., van de Vondervoort, P.J.I., de Graaff, L.H., 2002. EglC, a new endoglucanase from *Aspergillus niger* with major activity towards xyloglucan. Appl. Environ. Microbiol. 68, 1556–1560. https://doi.org/10.1128/AEM.68.4.1556-1560.2002.

Hassan, L., Lin, L., Sorek, H., Sperl, L., Goudoulas, T., Hagn, F., Germann, N., Tian, C., Benz, J., 2019. Crosstalk of cellulose and mannan perception pathways leads to inhibition of cellulase production in several filamentous fungi. MBio 10, e00277–e00319. https://doi.org/10.1128/MBIO.00277-19.

Karr-Lilienthal, L.K., Kadzere, C.T., Grieshop, C.M., Fahey, G.C., 2005. Chemical and nutritional properties of soybean carbohydrates as related to nonruminants: A review. Livest. Prod. Sci. 97, 1–12. https://doi.org/10.1016/J.LIVPRODSCI.2005.01.015.

Kowalczyk, J.E., Lubbers, R.J.M., Peng, M., Battaglia, E., Visser, J., de Vries, R.P., 2017. Combinatorial control of gene expression in *Aspergillus niger* grown on sugar beet pectin. Sci. Rep. 7, 12356. https://doi.org/10.1038/s41598-017-12362-y.

Kun, R.S., Garrigues, S., Di Falco, M., Tsang, A., de Vries, R.P., 2021. Blocking utilization of major plant biomass polysaccharides leads *Aspergillus niger* towards utilization of minor components. Microb. Biotechnol. 14, 1683–1698. https://doi.org/10.1111/1751-7915.13835.

Kunitake, E., Kobayashi, T., 2017. Conservation and diversity of the regulators of cellulolytic enzyme genes in Ascomycete fungi. Curr. Genet. 63, 951–958. https://doi.org/10.1007/S00294-017-0695-6.

Li, N., Kunitake, E., Aoyama, M., Ogawa, M., Kanamaru, K., Kimura, M., Koyama, Y., Kobayashi, T., 2016. McmA-dependent and -independent regulatory systems governing expression of ClrB-regulated cellulase and hemicellulase genes in *Aspergillus nidulans*. Mol. Microbiol. 102, 810–826. https://doi.org/10.1111/mmi.13493.

Li, Z., Yao, G., Wu, R., Gao, L., Kan, Q., Liu, M., Yang, P., Liu, G., Qin, Y., Song, X., Zhong, Y., Fang, X., Qu, Y., 2015. Synergistic and dose-controlled regulation of cellulase gene expression in *Penicillium oxalicum*. PLoS Genet. 11, e1005509.

Lin, H., Zhao, J., Zhang, Q., Cui, S., Fan, Z., Chen, H., Tian, C., 2020. Identification and characterization of a cellodextrin transporter in *Aspergillus niger*. Front. Microbiol. 11, 145. https://doi.org/10.3389/FMICB.2020.00145.

Liu, H.-M., Li, H.-Y., 2017. Application and conversion of soybean hulls, in: Kasai, M. (Ed.), Soybean - The Basis of Yield, Biomass and Productivity. IntechOpen, pp. 111–132. https://doi.org/10.5772/66744.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M., Henrissat, B., 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42, D490–D495. https://doi.org/10.1093/nar/gkt1178.

Love, M., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/S13059-014-0550-8.

Malgas, S., van Dyk, S.J., Pletschke, B.I., 2015. β-mannanase (Man26A) and α-galactosidase (Aga27A) synergism - a key factor for the hydrolysis of galactomannan substrates. Enzyme Microb. Technol. 70, 1–8. https://doi.org/10.1016/J.ENZMICTEC.2014.12.007.

Meyer, V., Arentshorst, M., El-Ghezal, A., Drews, A.C., Kooistra, R., van den Hondel, C.A. M.J.J., Ram, A.F.J., 2007. Highly efficient gene targeting in the *Aspergillus niger kusA* mutant. J. Biotechnol. 128, 770–775. https://doi.org/10.1016/j.jbiotec.2006.12.021.

Mudgil, D., Barak, S., Khatkar, B.S., 2014. Guar gum: processing, properties and food applications-A Review. J. Food Sci. Technol. 51, 409–418. https://doi.org/10.1007/S13197-011-0522-X.

Ogawa, M., Kobayashi, T., Koyama, Y., 2012. ManR, a novel $Zn(II)_2Cys_6$ transcriptional activator, controls the β-mannan utilization system in *Aspergillus oryzae*. Fungal Genet. Biol. 49, 987–995. https://doi.org/10.1016/j.fgb.2012.09.006.

Ogawa, M., Kobayashi, T., Koyama, Y., 2013. ManR, a transcriptional regulator of the β-mannan utilization system, controls the cellulose utilization system in *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. 77, 426–429. https://doi.org/10.1271/bbb.120795.

Peng, M., Aguilar-Pontes, M., de Vries, R., Mäkelä, M., 2018. In silico analysis of putative sugar transporter genes in *Aspergillus niger* using phylogeny and comparative transcriptomics. Front. Microbiol. 9, 1045. https://doi.org/10.3389/FMICB.2018.01045.

Raulo, R., Kokolski, M., Archer, D.B., 2016. The roles of the zinc finger transcription factors XlnR, ClrA and ClrB in the breakdown of lignocellulose by *Aspergillus niger*. AMB Express 6, 5. https://doi.org/10.1186/s13568-016-0177-0.

Rodiek, A.V., Stull, C.L., 2007. Glycemic index of ten common horse feeds. J. Equine Vet. Sci. 27, 205–211. https://doi.org/10.1016/J.JEVS.2007.04.002.

Samal, A., Craig, J.P., Coradetti, S.T., Philipp Benz, J., Eddy, J.A., Price, N.D., Glass, N.L., 2017. Network reconstruction and systems analysis of plant cell wall deconstruction by *Neurospora crassa*. Biotechnol. Biofuels 10, 225. https://doi.org/10.1101/133165.

Thomas-Chollier, M., Sand, O., Turatsinze, J.V., Janky, R., Defrance, M., Vervisch, E., Brohée, S., van Helden, J., 2008. RSAT: regulatory sequence analysis tools. Nucleic Acids Res. 36, W119–W127. https://doi.org/10.1093/nar/gkn304.

Thombare, N., Jha, U., Mishra, S., Siddiqui, M., 2016. Guar gum as a promising starting material for diverse applications: A review. Int. J. Biol. Macromol. 88, 361–372. https://doi.org/10.1016/J.IJBIOMAC.2016.04.001.

van Munster, J., Daly, P., Blythe, M., Ibbett, R., Kokolski, M., Gaddipati, S., Lindquist, E., Singan, V., Barry, K., Lipzen, A., Ngan, C., Petzold, C., Chan, L., Arvas, M., Raulo, R., Pullan, S., Delmas, S., Grigoriev, I., Tucker, G., Simmons, B., Archer, D., 2020. Succession of physiological stages hallmarks the transcriptomic response of the fungus *Aspergillus niger* to lignocellulose. Biotechnol. Biofuels 13, 69. https://doi.org/10.1186/S13068-020-01702-2.

van Peij, N.N.M.E., Gielkens, M.M.C., de Vries, R.P., Visser, J., de Graaff, L.H., 1998a. The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. Appl. Environ. Microbiol. 64, 3615–3619. https://doi.org/10.1128/aem.64.10.3615-3619.1998.

van Peij, N.N.M.E., Visser, J., de Graaff, L.H., 1998b. Isolation and analysis of *xlnR*, encoding a transcriptional activator co-ordinating xylanolytic expression in *Aspergillus niger*. Mol. Microbiol. 27, 131–142. https://doi.org/10.1046/j.1365-2958.1998.00666.x.

Whistler, R.L., Saarnio, J., 1957. Galactomannan from soy bean hulls. J. Am. Chem. Soc. 79, 6055–6057. https://doi.org/10.1021/ja01579a057.