microbial biotechnology

Blocking utilization of major plant biomass polysaccharides leads *Aspergillus niger* towards utilization of minor components

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Summary

Fungi produce a wide range of enzymes that allow them to grow on diverse plant biomass. Wheat bran is a low-cost substrate with high potential for biotechnological applications. It mainly contains cellulose and (arabino)xylan, as well as starch, proteins, lipids and lignin to a lesser extent. In this study, we dissected the regulatory network governing wheat bran degradation in *Aspergillus niger* to assess the relative contribution of the regulators to the utilization of this plant biomass substrate. Deletion of genes encoding transcription factors involved in (hemi-)cellulose utilization (XlnR, AraR, ClrA and ClrB) individually and in combination significantly reduced production of polysaccharide-degrading enzymes, but retained substantial growth on wheat bran. Proteomic analysis suggested the ability of *A. niger* to grow on other carbon components, such as starch, which was confirmed by the additional deletion of the amylolytic regulator AmyR. Growth was further reduced but not impaired, indicating that other minor components provide sufficient energy for residual growth, displaying the flexibility of *A. niger*, and likely other fungi, in carbon utilization.

Better understanding of the complexity and flexibility of fungal regulatory networks will facilitate the generation of more efficient fungal cell factories that use plant biomass as a substrate.

Introduction

Plant biomass is the most abundant carbon source on Earth and mainly consists of plant cell wall polymers (cellulose, hemicellulose, pectin and lignin) (de Vries and Visser, 2001). In nature, filamentous fungi, such as *Aspergillus niger*, secrete large arrays of hydrolytic enzymes to degrade the aforementioned polymers. Fungal Carbohydrate-Active enZymes (CAZymes) are used in many industrial sectors for the production of pulp and paper, food and feed, detergents, textiles, and biofuels and biochemicals (Mäkelä *et al.*, 2014). In this context, low-cost substrates are of high interest for many biotechnological applications. Wheat bran, a by-product of wheat milling, is the outer layer of wheat grain. It contains mainly cellulose and (arabino)xylan, as can be seen from the total sugar composition (Table S1). Wheat bran also contains starch, mixed-linked β-D-glucans (including xyloglucan), as well as lignin, proteins and small amounts of lipids (DuPont and Selvendran, 1987; Stevens and Selvendran, 1988; Parker *et al.*, 2005; Ruthes *et al.*, 2017; Rudjito *et al.*, 2019).

Transcriptional regulators or transcription factors play a key role in plant biomass degradation by fungi as they control the expression and synthesis of enzymes required for the degradation of different plant polysaccharides. The regulation system governed by transcription factors ensures that only those enzymes that are needed to degrade the prevalent substrate will be produced to avoid wasting energy on the production of enzymes that are not required. Several fungal transcription factors involved in plant biomass degradation have been identified across industrial species and fungal reference species (Benocci *et al.*, 2017).

The first identified (hemi-)cellulolytic regulator is the *A. niger* XlnR (van Peij *et al.*, 1998b), and orthologs have been widely studied in other fungal species (Benocci *et al.*, 2017), highlighting the key role of XlnR in the process of cellulose and hemicellulose utilization in fungi. Another hemicellulolytic transcription factor, AraR,

controls the arabinanolytic system (Battaglia *et al.*, 2011), and together with XlnR, controls the pentose catabolic pathway. The pentose catabolic pathway is required for the utilization of two main monomeric sugars found in hemicellulose, D-xylose and L-arabinose (Battaglia *et al.*, 2014). In addition, the transcription factors Clr-1 and Clr-2 were identified in *Neurospora crassa*, and their corresponding gene deletions were shown to result in impaired cellulolytic activities (Coradetti *et al.*, 2012). So far, Clr-1 homologs showing conserved function have been reported for *A. nidulans* (Coradetti *et al.*, 2012) and *A. niger* (ClrA) (Raulo *et al.*, 2016) while Clr-2 homologs have been described for multiple species, such as *A. nidulans* (Coradetti *et al.*, 2013), *A. niger* (Raulo *et al.*, 2016), *Penicillium oxalicum* (Yao *et al.*, 2015) (ClrB) and *A. oryzae* (Ogawa *et al.*, 2013). In *A. oryzae,* the Clr-2 homolog was initially described as a regulator of β-mannan utilization (ManR) (Ogawa *et al.*, 2012), but whether it regulates mannan degradation in the other species has not been reported. In *A. niger*, ClrB plays a more dominant role in cellulose utilization compared with ClrA and both appear to be influenced by XlnR (Raulo *et al.*, 2016). Considering the composition of wheat bran, these four transcription factors are expected to have a major role in its degradation by *A. niger*, while other transcription factors, such as AmyR (Petersen *et al.*, 1999) (starch degradation), InuR (Yuan *et al.*, 2008a) (inulin degradation) and GaaR (Alazi *et al.*, 2016), RhaR (Gruben *et al.*, 2014) and GalX (Kowalczyk *et al.*, 2015) (pectin degradation), are expected to have no or a minor role.

In this study, we used CRISPR/Cas9 genome editing (Song *et al.*, 2018) to generate a set of *A. niger* XlnR-AraR-ClrA-ClrB deletion mutants to assess their relative contribution to wheat bran degradation and identify other possible transcription factors involved in wheat bran utilization by this fungus. The characterization of mutants carrying individual and combinatorial deletions of key transcription factors helps our understanding of the complexity of the regulatory network involved in the degradation of a crude substrate. This knowledge can facilitate the generation of fungal cell factories with high industrial applicability that use plant biomass as a substrate, through targeted engineering of the regulatory system.

Results

Combined deletion of xlnR, araR, clrA and clrB does not impair growth on wheat bran

Null mutations of XlnR, AraR and ClrA reduced growth on wheat bran, while ClrB-null resulted in improved growth on this substrate (Fig. 1A and Fig. S1). The improved growth of ClrB-null was also observed in strains with combined deletions, but mainly when XlnR

remained present in the strain. All other combined deletion strains showed reduced growth on wheat bran, but significant residual growth remained in all strains. To better capture the influence of the different regulators, growth was also evaluated on the polymeric and monomeric components of wheat bran (Fig. 1A). Deletion of *xlnR* or *clrB* abolished growth on cellulose, while Δ*clrA* and Δ*araR* mutants displayed reduced and normal growth, respectively. Only deletion of *xlnR* affected growth on xylan. While growth on xyloglucan was mostly affected by deletion of *araR* or *xlnR*, Δ*clrB* mutant only showed growth reduction at initial stages of growth on this substrate, and growth ability was recovered over time. No growth reduction was observed for any of the mutants on maltose, starch or cellobiose. Growth differences on D-xylose and L-arabinose reflect the influence of XlnR and AraR on sugar catabolism. Growth on D-xylose was abolished in strains in which both *xlnR* and *araR* were deleted, but only minor differential growth phenotype compared with the control strain was observed in the other mutant strains. Growth on L-arabinose was reduced in strains where *araR* was deleted, with a stronger reduction if *xlnR* was also absent.

Overall, the strongest growth reduction on wheat bran was observed in strains in which *xlnR* was deleted in combination with any of the other three transcription factors, especially after six days of growth. The Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* mutant (subsequently referred to as quadruple mutant) was still able to grow on wheat bran, suggesting that it was utilizing other components, such as starch, a polysaccharide on which none of the tested mutants showed any differential phenotype.

The relative growth reduction in the mutant strains correlates with reduced levels of key enzyme activities

To evaluate whether the reduced growth shown by the different mutant strains (Fig. 1A) could be a direct result of reduced enzyme levels, samples of liquid cultures containing 3% wheat bran were first analysed by SDS-PAGE (Fig. 1B). Deletion of *xlnR* had the highest impact on the overall amount of extracellular protein, although in the quadruple mutant protein production was further reduced. Activity measurements for some relevant enzymes (Data S1A and B) were performed to provide insight into the molecular mechanisms that underlie the phenotypic differences between the strains.

The abolished growth of Δ*xlnR* on cellulose (Fig. 1A) correlates with the reduction in β-1,4-D-glucosidase (BGL) activity in this strain (Fig. 1C), which is crucial for the release of D-glucose units from cellulose or cellobiose. However, both growth and BGL activity suggest that ClrA is less involved in the regulation of cellulose degradation
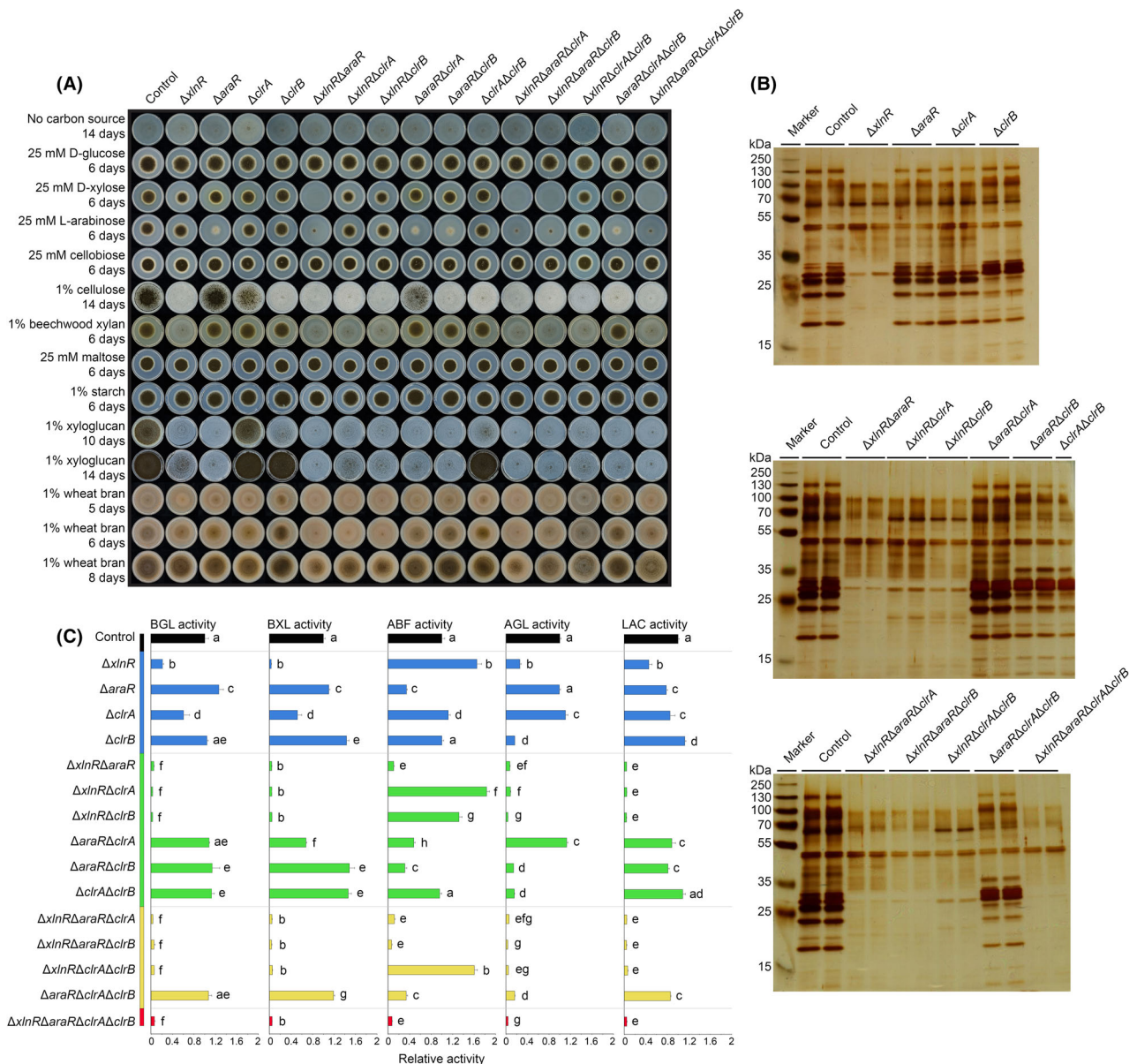
Fig. 1. Characterization of *A. niger* XlnR-AraR-ClrA-ClrB deletion mutants.
A. Growth profile of *A. niger* control and mutant strains. Selected carbon sources were inoculated with 1000 spores and incubated at 30°C for up to 14 days.
B. Extracellular protein production of *A. niger* control and mutant strains analysed by SDS-PAGE after 24 h of growth on 3% wheat bran liquid cultures. Supernatant samples are analysed in biological duplicates.
C. Enzyme activity assays of the supernatants from *A. niger* control and mutant strains. The control, and the single-, double-, triple- and quadruple mutant strains are indicated by different colours. Data represents the normalized mean values of biological duplicates and technical triplicates and the standard deviation. The absorbance values measured at 405 nm and the amount of released *p*-nitrophenol by each strain are described in Data S1B. BGL = β-1,4-D-glucosidase, BXL = β-1,4-xylosidase, ABF = α-L-arabinofuranosidase, AGL = α-1,4-D-galactosidase, LAC = β-1,4-D-galactosidase activity. Letters (a–h) are shown to explain the statistical differences between samples within each specific enzyme assay. Samples showing different letters show significant differences among the strains within each specific enzyme assay, while samples sharing the same letters show no statistically significant differences (ANOVA and Tukey's HDS test, $>P < 0.05$).

than XlnR in *A. niger*. The abolished growth of Δ*clrB* on cellulose cannot be explained by reduced BGL activity, suggesting that ClrB is more important for the production of cellobiohydrolases and/or endoglucanases (Raulo

et al., 2016), which are crucial enzymes for degradation of cellulose.

D-xylose release from (arabino)xylan, which is one of the most abundant polysaccharides of wheat bran, is

catalysed by β-xylosidase (BXL) activity. Similar to BGL activity, deletion of *xlnR* showed the highest impact on BXL activity (Fig. 1C), which was abolished in every mutant in which *xlnR* was deleted, and which correlated with minimal growth of these strains on beechwood xylan (Fig. 1A). Contrary to Δ*xlnR*, the Δ*araR* and Δ*clrB* mutants showed increased levels of BXL activity. However, they did not lead to increased growth on beechwood xylan. The BGL and BXL activities together with the growth profile indicate that XlnR has the overall highest impact on cellulose and (arabino)xylan utilization.

There was limited correlation between the levels of three accessory enzymes (α-L-arabinofuranosidase (ABF), α-1,4-D-galactosidase (AGL) and β-1,4-D-galactosidase (LAC), involved in degradation of several plant cell wall polysaccharides) (Fig. 1C) and the growth profile (Fig. 1A). However, the deletion mutants revealed which regulators affect the production of these enzymes. The Δ*clrA* mutant did not show decreased ABF or AGL activity while the reduction in LAC activity was minimal, indicating that ClrA does not control the genes encoding ABF, AGL or LAC. The Δ*xlnR* mutant showed significant reduction in AGL and LAC activities while deletion of *araR* significantly reduced ABF activity, and to a lesser extent LAC activity. Finally, deletion of *clrB* resulted in decreased AGL activity. Overall, the results confirm the regulatory roles of XlnR, AraR and ClrB on the genes encoding the enzymes responsible for the three accessory activities. Since these enzymatic activities are important for the degradation of xyloglucan, the results match the reduced ability of Δ*xlnR* and Δ*araR* to grow on xyloglucan. As ABF, AGL and LAC activities contribute to growth on xylan, the abolished enzymatic activities in the quadruple mutant in all tested conditions correlate with the abolished growth on cellulose, xylan and xyloglucan.

Residual starch in wheat bran explains the limited growth reduction in the quadruple mutant

Proteomics of selected samples (Data S2A) was performed to analyse in depth the effect of regulatory mutations on the production of individual plant biomass degrading enzymes in *A. niger*. The high amount of (arabino)xylan in wheat bran resulted in an abundant presence of xylanolytic enzymes (24.24% of the total exoproteome), as well as cellulolytic enzymes (four cellobiohydrolases and one BGL) in the control strain (Fig. 2 and Data S2B). However, the two detected endoglucanases represented only 0.16% (EglC) and 0.03% (EglB) (Data S2A) of the total exoproteome, correlating with the slow utilization of cellulose by *A. niger*.

Of the single deletion mutants, Δ*xlnR* showed the highest reduction in CAZymes, which is also reflected in the SDS-PAGE (Fig. 1B) and enzyme activity (Fig. 1C) results. The minimal presence of (arabino)xylan-acting

enzymes in the Δ*xlnR* supernatant was similar to that of the quadruple mutant, although the overall abundance of cellulolytic enzymes was further reduced in the supernatant of the quadruple mutant (Fig. 2). Binding site analysis confirmed that all genes encoding the (arabino)xylanolytic and cellulolytic enzymes affected by the deletion of *xlnR* carry the putative XlnR binding site (GGCTAR) (de Vries *et al.*, 2002) in their promoter sequences (Table S2), thus suggesting direct regulation of these genes by XlnR.

The quadruple deletion mutant showed further reduction in the abundance of two cellobiohydrolases (CbhA and CbhB), an acetylesterase (HaeA) and a β-1,4-endo-mannanase (ManA) compared with the Δ*xlnR* mutant (Fig. 2, Cluster C). These are all enzymes that are negatively affected by the single *clrB* deletion, and the promoter sequences of their corresponding genes contain the putative ClrB binding site (CGGN$_8$CCG) (Li *et al.*, 2016) (Table S2), suggesting direct regulation of the Cluster C genes by ClrB. A broad range of other CAZymes showed highly reduced abundance or absence in the quadruple mutant compared with any single deletion strain (Fig. 2), suggesting combinatorial control by the studied transcription factors.

Amylases are the major carbohydrate-degrading enzymes present in the secretome of the quadruple mutant (Data S2B). This correlates with the residual starch present in washed wheat bran (as described in Experimental procedures), which most likely has become the only carbohydrate that supports the growth of the quadruple mutant. Furthermore, nearly all proteins in the quadruple mutant decrease in abundance by at least twofold (Fig. 2). Among the few proteins that do not decrease in abundance in the quadruple mutant are three enzymes: a glucoamylase (GlaA), an α-amylase (AamA) and an α-1,4-galactosidase (AglA). The genes encoding these enzymes have been shown to be regulated by the amylolytic transcription factor AmyR (Fowler *et al.*, 1990; Yuan *et al.*, 2008b; Coutinho *et al.*, 2009; vanKuyk *et al.*, 2012; Gruben *et al.*, 2017).

To confirm the use of starch by the quadruple mutant during growth on wheat bran, we deleted *amyR* in this mutant and compared the phenotype to that of the quadruple mutant and the single Δ*amyR* strain. The Δ*amyR* mutant was unable to grow on maltose and starch, but growth on wheat bran was not affected after 6, 8 or 10 days (Fig. 3A). This unaltered growth suggests that the starch found in washed wheat bran contributes little to the growth of *A. niger*. In contrast, the Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* mutant (subsequently referred to as quintuple mutant) showed a strong growth reduction compared with the quadruple mutant (Fig. S2). This result demonstrates the ability of *A. niger* to maintain growth by using the starch component of wheat
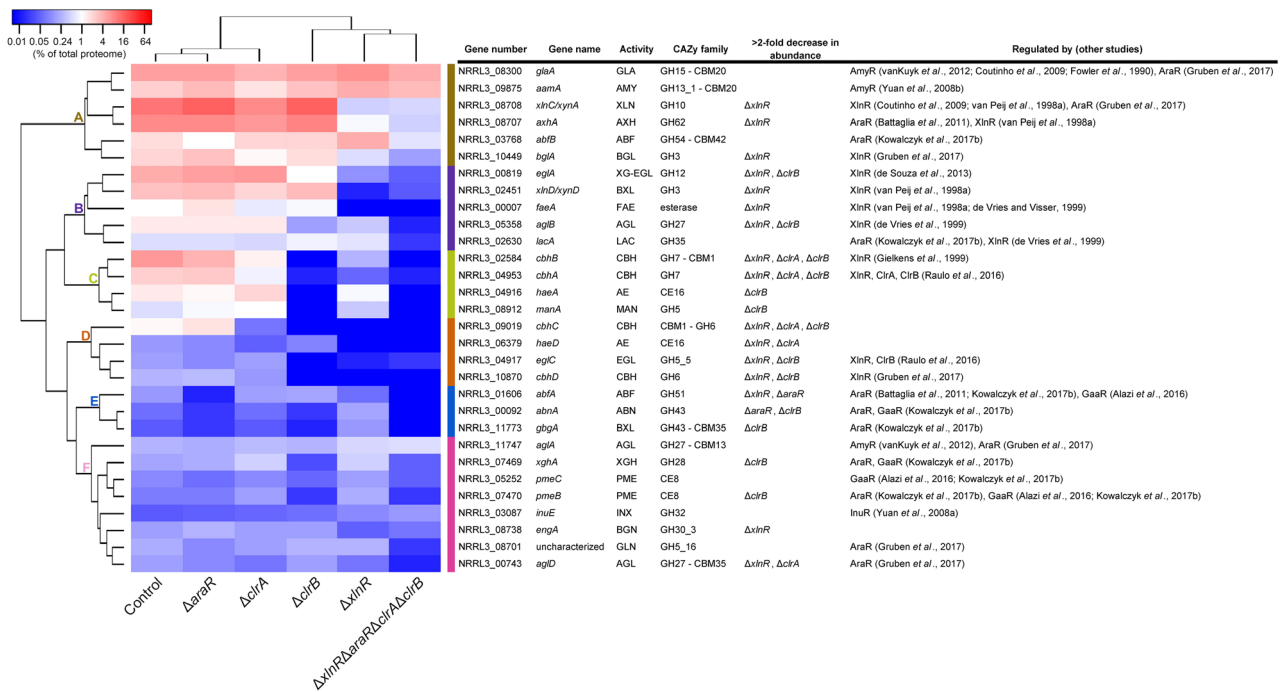
Gene number	Gene name	Activity	CAZy family	>2-fold decrease in abundance	Regulated by (other studies)
NRRL3_08300	glaA	GLA	GH15 - CBM20		AmyR (vanKuyk et al., 2012; Coutinho et al., 2009; Fowler et al., 1990), AraR (Gruben et al., 2017)
NRRL3_09875	aamA	AMY	GH13_1 - CBM20		AmyR (Yuan et al., 2008b)
NRRL3_08708	xlnC/xynA	XLN	GH10	ΔxlnR	XlnR (Coutinho et al., 2009; van Peij et al., 1998a), AraR (Gruben et al., 2017)
NRRL3_08707	axhA	AXH	GH62	ΔxlnR	AraR (Battaglia et al., 2011), XlnR (van Peij et al., 1998a)
NRRL3_03768	abfB	ABF	GH54 - CBM42		AraR (Kowalczyk et al., 2017b)
NRRL3_10449	bglA	BGL	GH3	ΔxlnR	XlnR (Gruben et al., 2017)
NRRL3_00819	eglA	XG-EGL	GH12	ΔxlnR, ΔclrB	XlnR (de Souza et al., 2013)
NRRL3_02451	xlnD/xynD	BXL	GH3	ΔxlnR	XlnR (van Peij et al., 1998a)
NRRL3_00007	faeA	FAE	esterase	ΔxlnR	XlnR (van Peij et al., 1998a; de Vries and Visser, 1999)
NRRL3_05358	aglB	AGL	GH27	ΔxlnR, ΔclrB	XlnR (de Vries et al., 1999)
NRRL3_02630	lacA	LAC	GH35		AraR (Kowalczyk et al., 2017b), XlnR (de Vries et al., 1999)
NRRL3_02584	cbhB	CBH	GH7 - CBM1	ΔxlnR, ΔclrB	XlnR (Gielkens et al., 1999)
NRRL3_04953	cbhA	CBH	GH7	ΔxlnR, ΔclrA, ΔclrB	XlnR, ClrA, ClrB (Raulo et al., 2016)
NRRL3_04916	haeA	AE	CE16	ΔclrB	
NRRL3_08912	manA	MAN	GH5	ΔclrB	
NRRL3_09019	cbhC	CBH	CBM1 - GH6	ΔxlnR, ΔclrA, ΔclrB	
NRRL3_06379	haeD	AE	CE16	ΔxlnR, ΔclrA	
NRRL3_04917	eglC	EGL	GH5_5	ΔxlnR, ΔclrB	XlnR, ClrB (Raulo et al., 2016)
NRRL3_10870	cbhD	CBH	GH6	ΔxlnR, ΔclrB	XlnR (Gruben et al., 2017)
NRRL3_01606	abfA	ABF	GH51	ΔxlnR, ΔaraR	AraR (Battaglia et al., 2011; Kowalczyk et al., 2017b), GaaR (Alazi et al., 2016)
NRRL3_00092	abnA	ABN	GH43	ΔaraR, ΔclrB	AraR, GaaR (Kowalczyk et al., 2017b)
NRRL3_11773	gbgA	BXL	GH43 - CBM35	ΔclrB	AraR (Kowalczyk et al., 2017b)
NRRL3_11747	aglA	AGL	GH27 - CBM13		AmyR (vanKuyk et al., 2012), AraR (Gruben et al., 2017)
NRRL3_07469	xghA	XGH	GH28	ΔclrB	AraR, GaaR (Kowalczyk et al., 2017b)
NRRL3_05252	pmeC	PME	CE8		GaaR (Alazi et al., 2016; Kowalczyk et al., 2017b)
NRRL3_07470	pmeB	PME	CE8	ΔclrB	AraR (Kowalczyk et al., 2017b), GaaR (Alazi et al., 2016; Kowalczyk et al., 2017b)
NRRL3_03087	inuE	INX	GH32		InuR (Yuan et al., 2008a)
NRRL3_08738	engA	BGN	GH30_3	ΔxlnR	
NRRL3_08701	uncharacterized	GLN	GH5_16		AraR (Gruben et al., 2017)
NRRL3_00743	aglD	AGL	GH27 - CBM35	ΔxlnR, ΔclrA	AraR (Gruben et al., 2017)

Fig. 2. Hierarchical clustering of CAZymes found in the supernatant of *A. niger* control and Δ*xlnR*, Δ*araR*, Δ*clrA*, Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* mutant strains. The 24 h supernatant samples originated from 3% wheat bran liquid cultures. Colour code represents the averaged percentage value of the total exoproteome of duplicate samples. Regulation of genes is based on previous studies (Fowler *et al.*, 1990; van Peij *et al.*, 1998a; de Vries and Visser, 1999; de Vries *et al.*, 1999; Gielkens *et al.*, 1999; Yuan *et al.*, 2008a,b; Coutinho *et al.*, 2009; Battaglia *et al.*, 2011; vanKuyk *et al.*, 2012; de Souza *et al.*, 2013; Alazi *et al.*, 2016; Raulo *et al.*, 2016; Gruben *et al.*, 2017; Kowalczyk *et al.*, 2017b). Enzyme abbreviations are described in Table S7. Enzymes with a lower abundance than 0.1% of the total proteome in each sample were excluded from the analysis. The complete exoproteome data are described in Data S2A.

bran, when utilization of the major carbohydrates is blocked.

The ΔxlnRΔaraRΔclrAΔclrBΔamyR mutant utilizes residual carbon sources found in wheat bran

Proteomics of liquid culture samples of the control, quadruple and quintuple mutant strains (Data S3A) demonstrated a further reduced protein production profile for the quintuple mutant compared with the quadruple strain due to the lack of amylolytic enzymes (Fig. 3B). The CAZyme content in the supernatant of the quintuple mutant (Fig. 3C) reduced to only 1.61% of the total exoproteome (Data S3B). The abundance of the single detected glucoamylase (GlaA) and the major α-amylase (AamA) in the quadruple mutant was strongly reduced when *amyR* was deleted (Fig. 3C, cluster B). Moreover, the α-1,4-D-glucosidase (AGD) and glucoamylase (GLA) activities involved in starch degradation have also been abolished in the quintuple mutant (Fig. S3 and Data S1C). These results suggest that the quintuple mutant is not able to utilize starch, which explains the observed further growth reduction on wheat bran (Fig. 3A). Interestingly, the abundance of an arabinoxylan arabinofuranohydrolase (AxhA) (Fig. 3C, cluster A) and an exo-inulinase (InuE) (Fig. 3C, cluster C) was also reduced more

than twofold compared with the quadruple strain. Analyses of the promoter sequences of their corresponding genes revealed that both carry putative AmyR binding sites (Table S2), suggesting that AmyR may be involved in the regulation of these genes.

The exoproteome of the quintuple mutant contained a broad range of proteases that were also observed in the other strains. Due to the reduction in CAZymes, the relative contribution of non-CAZy proteins increased from 40.32% in the control strain to 98.39% in the quintuple strain (Fig. 4A and Data S3B). In all strains, the most abundant non-CAZy protein was the aspartic peptidase PepA (Fig. 4B, cluster A), which is under the control of the transcription factor PrtT, a specific regulator of extracellular proteases in filamentous fungi (Punt *et al.*, 2008). PepA, together with other PrtT-controlled proteases (ProtA, ProtB, NRRL3_11745, PepF, NRRL3_05873 and NRRL3_01776) (Huang *et al.*, 2020), showed a relative increase in abundance when the (hemi-)cellulolytic enzyme system was impaired in the quadruple and quintuple deletion mutants (Fig. 4B and Data S2A and Data S3A). Lipases were detected in very small amounts, with only one (LipanI) detected at > 0.1% abundance in all strains (Fig. 4B, cluster B), but with its highest abundance (1.95%) in the quintuple mutant

(A)

(B)

(C)
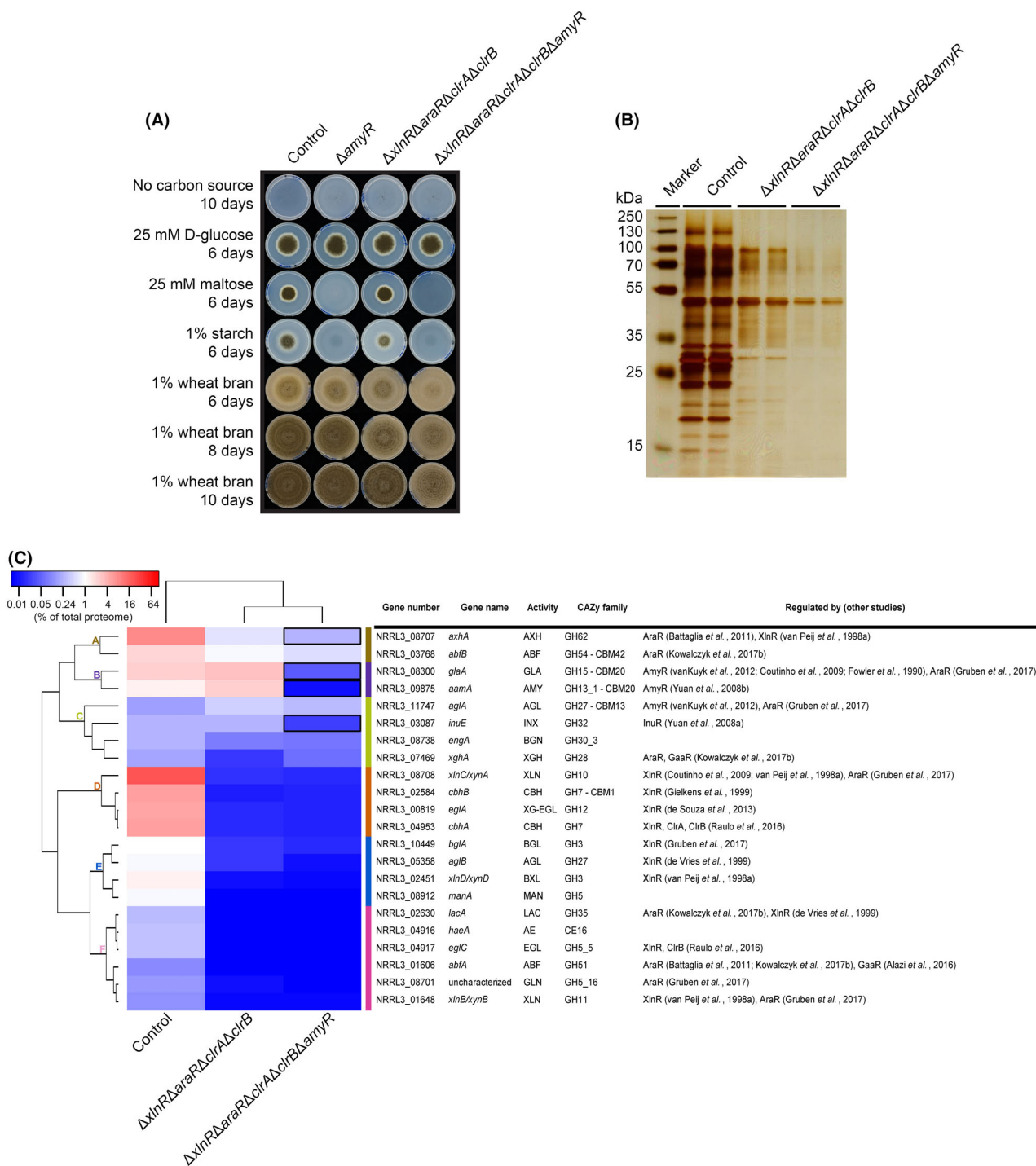
(Data S3A). Overall, these results indicate that the quintuple mutant utilizes proteins as a primary carbon source when carbohydrate catabolism is blocked.

Predicted intracellular proteins are found in very low quantities in the secretome (Table S3), indicating that cell lysis did not occur extensively in our experiments. Moreover, the most abundant putative intracellular protein (NRRL3_00054) was also present in the control strain (Table S3), suggesting that the presence of some intracellular proteins may be linked to the experimental condition rather than to the deletion of transcription factors and poor growth. Overall, these results suggest that *A. niger* is able to survive on wheat bran after 24 h incubation by utilizing residual carbon sources when carbohydrate utilization is blocked.

Fig. 3. Characterization of *A. niger* Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* quintuple deletion mutant.
A. Growth profile of *A. niger* control, and the Δ*amyR*, Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* mutant strains. Selected carbon sources were inoculated with 1000 spores and incubated at 30°C for up to 10 days.
B. Extracellular protein production of *A. niger* control and mutant strains analysed by SDS-PAGE. The 24 h supernatant samples originated from 3% wheat bran liquid cultures. Samples are analysed in biological duplicates.
C. Hierarchical clustering of CAZymes found in the supernatant of *A. niger* control, and the Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* mutant strains. Colour code represents averaged percentage value of the total exoproteome of duplicate samples. Black rectangles indicate CAZymes from the Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* mutant showing > two fold decrease in abundance compared with the Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* strain. Regulation of genes is based on previous studies (Fowler *et al.*, 1990; van Peij *et al.*, 1998a; de Vries *et al.*, 1999; Gielkens *et al.*, 1999; Yuan *et al.*, 2008a,b; Coutinho *et al.*, 2009; Battaglia *et al.*, 2011; vanKuyk *et al.*, 2012; de Souza *et al.*, 2013; Alazi *et al.*, 2016; Raulo *et al.*, 2016; Gruben *et al.*, 2017; Kowalczyk *et al.*, 2017b). Enzyme abbreviations are described in Table S7. Enzymes with a lower abundance than 0.1% of the total proteome in each sample were excluded from the analysis. The complete exoproteome data are described in Data S3A.
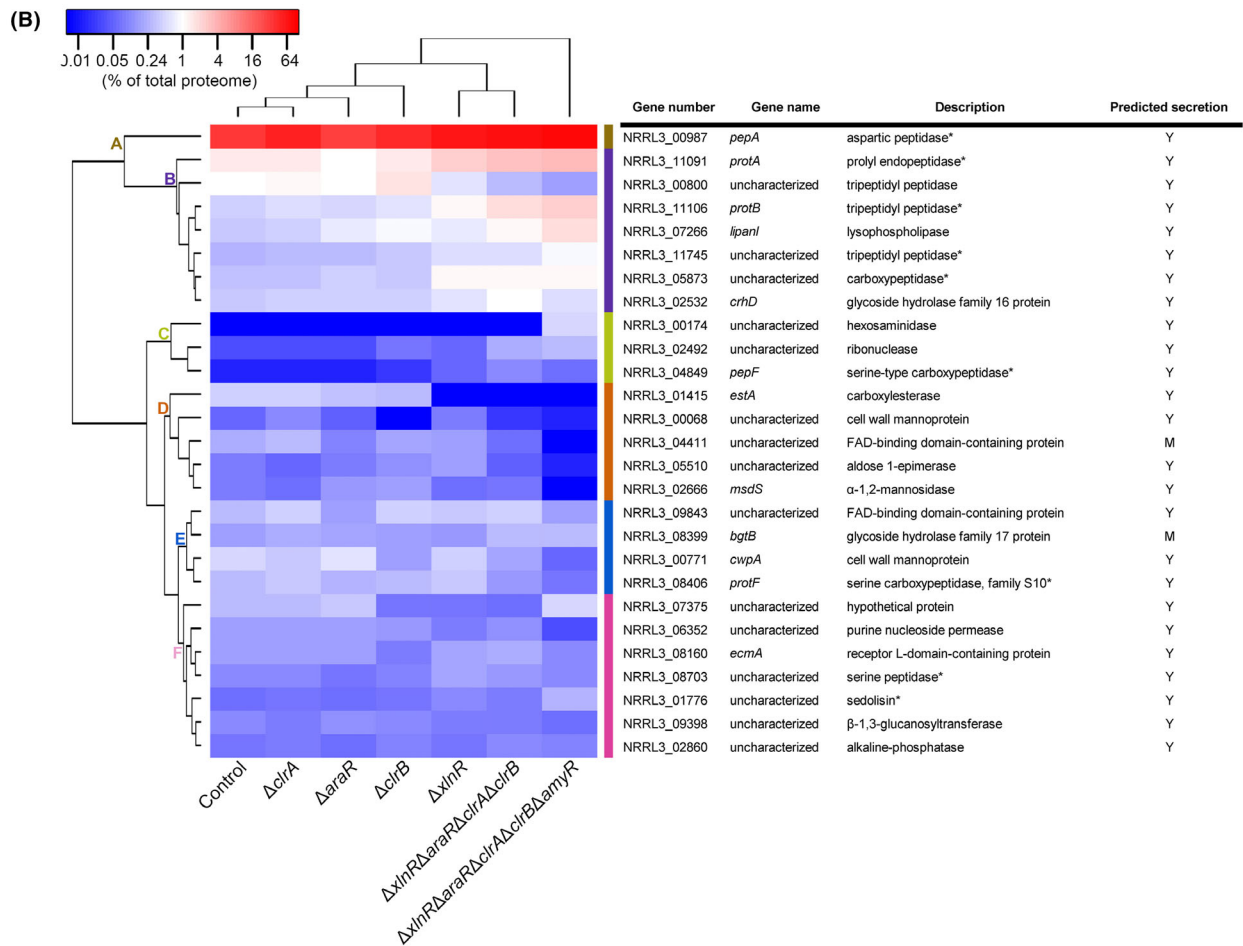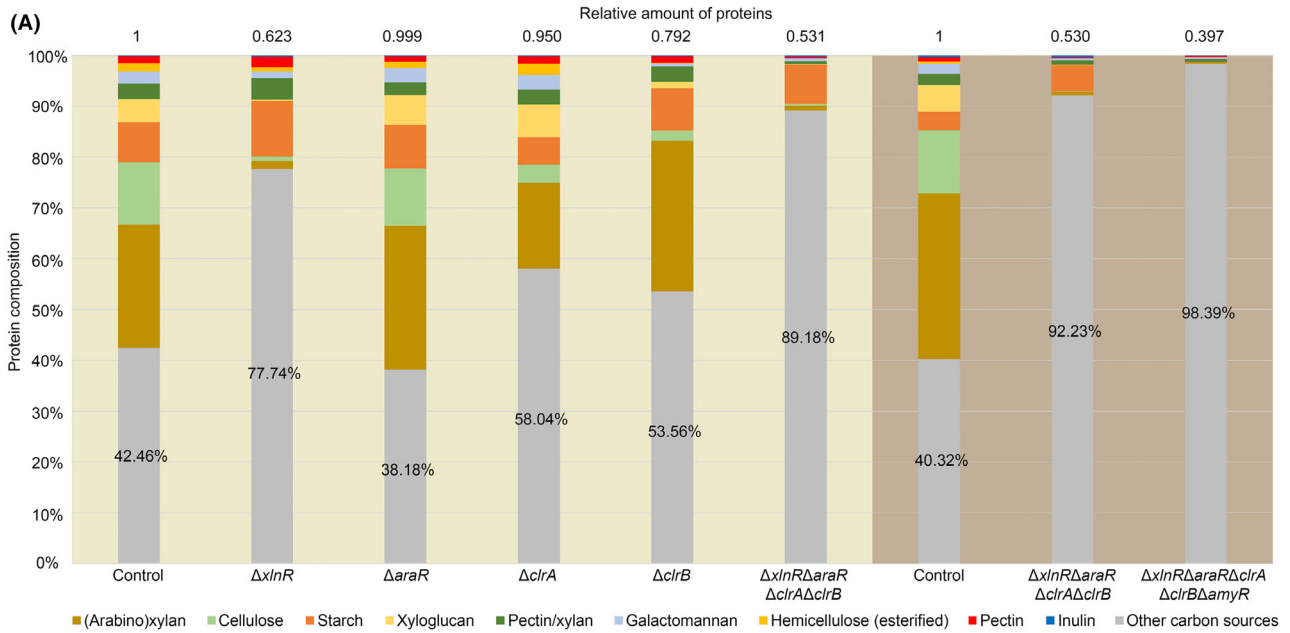
Discussion

The development of the biobased economy stimulates the development of fungal cell factories that convert plant biomass directly to desired products (e.g. proteins, metabolites) (Liaud *et al.*, 2015; Amores *et al.*, 2016). However, efficient design of such cell factories requires a detailed understanding of the plant biomass conversion process at the molecular level (Chroumpi *et al.*, 2021). Plant biomass conversion by fungi involves a complex system of transcriptional regulation to ensure that the right set of enzymes is produced that matches the composition of the prevailing substrate. Several transcription factors involved in this process have been identified in fungi, but their relative contribution, interaction and possible overlapping sets of target genes have not been addressed in detail. In the present study, we addressed these questions by performing an in-depth analysis of the contribution of five transcriptional activators involved in the conversion of wheat bran by *A. niger*.

Exoproteomics of the control strain revealed that *A. niger* degrades mainly (arabino)xylan. However, enzymes involved in cellulose degradation do not appear to be coordinately regulated under growth on wheat bran. Exo-acting cellulases are abundantly represented in the exoproteome while endoglucanases, which are essential for efficient degradation of cellulose, are detected at low levels. The low abundance of endoglucanases together with the slow growth on cellulose suggests that cellulose is not a preferred carbon source for *A. niger*. Lower levels of amylolytic and xyloglucanolytic enzymes were also detected, which correlates with the levels of xyloglucan and residual starch reported to be present in washed wheat bran (DuPont and Selvendran, 1987; Nyombaire, 2012). In addition, mannanolytic, pectinolytic and inulinolytic enzymes were detected, but based on the composition of wheat bran, it is not likely that galactomannan, pectin and inulin are present in sufficient amounts to support growth of *A. niger*. Nevertheless, trace amounts of the inducers for the production of these enzymes may explain their presence, as it was observed previously in a transcriptome study of *A. niger* during growth on guar gum (Coconi-Linares *et al.*, 2019).

Although the composition of fungal CAZymes is largely dependent on the incubation time and substrate composition, comparable results have been reported for the thermophilic fungus *Myceliophthora thermophila*. A combined transcriptome and exoproteome study showed mainly the upregulation of genes involved in xylan and cellulose degradation when grown on monocot plants (Kolbusz *et al.*, 2014). However, a comparative study of the *Trichoderma reesei* and *Talaromyces cellulolyticus* (formerly *Acremonium cellulolyticus*) secretomes showed differences in the major enzymatic activities (Fujii *et al.*, 2009). The supernatant derived from *T. cellulolyticus* showed higher cellulolytic activity and D-glucose yield from plant biomass, while the *T. reesei* supernatant showed higher xylanolytic activities than the supernatant of *T. cellulolyticus*. The synergistic action of xylanases and cellulases is necessary for the enzymatic degradation of various agricultural residues or woody substrates for biofuel production (Álvarez *et al.*, 2016).

The phenotype and exoproteome of the *A. niger* Δ*xlnR* mutant confirmed previous studies that reported impaired growth on cellulose, (arabino)xylan and xyloglucan (van Peij *et al.*, 1998a; Gruben *et al.*, 2017). Enzyme activity assays and proteomic studies also demonstrate the key role of XlnR in the utilization of (arabino)xylan and cellulose when grown on wheat bran, and correlate with a previous study that showed that colonization of wheat bran is mainly dependent on XlnR (Kowalczyk *et al.*, 2017a). However, it cannot be excluded that the phenotype of Δ*xlnR* includes reduced production of ClrA and ClrB targets, as it has previously been shown that XlnR affects the expression of the genes encoding these two transcription factors (Raulo *et al.*, 2016). Therefore, we conclude that XlnR is the dominant transcription factor for wheat bran utilization by *A. niger*.

Deletion of *clrA* has low impact on growth on the tested substrates. The significantly reduced BGL and BXL activity in the single Δ*clrA* mutant suggests that ClrA at least in part regulates the expression of their encoding genes. However, no reduced BGL activity was observed in the Δ*araR*Δ*clrA*, Δ*clrA*Δ*clrB* and Δ*araR*Δ*clrA*Δ*clrB*, while BXL activity was increased in the Δ*clrA*Δ*clrB* and Δ*araR*Δ*clrA*Δ*clrB* mutants. We therefore

(A)

Relative amount of proteins

| 1 | 0.623 | 0.999 | 0.950 | 0.792 | 0.531 | 1 | 0.530 | 0.397 |

■ (Arabino)xylan   ■ Cellulose   ■ Starch   ■ Xyloglucan   ■ Pectin/xylan   ■ Galactomannan   ■ Hemicellulose (esterified)   ■ Pectin   ■ Inulin   ■ Other carbon sources

(B)

Gene number	Gene name	Description	Predicted secretion
NRRL3_00987	pepA	aspartic peptidase*	Y
NRRL3_11091	protA	prolyl endopeptidase*	Y
NRRL3_00800	uncharacterized	tripeptidyl peptidase	Y
NRRL3_11106	protB	tripeptidyl peptidase*	Y
NRRL3_07266	lipanI	lysophospholipase	Y
NRRL3_11745	uncharacterized	tripeptidyl peptidase*	Y
NRRL3_05873	uncharacterized	carboxypeptidase*	Y
NRRL3_02532	crhD	glycoside hydrolase family 16 protein	Y
NRRL3_00174	uncharacterized	hexosaminidase	Y
NRRL3_02492	uncharacterized	ribonuclease	Y
NRRL3_04849	pepF	serine-type carboxypeptidase*	Y
NRRL3_01415	estA	carboxylesterase	Y
NRRL3_00068	uncharacterized	cell wall mannoprotein	Y
NRRL3_04411	uncharacterized	FAD-binding domain-containing protein	M
NRRL3_05510	uncharacterized	aldose 1-epimerase	Y
NRRL3_02666	msdS	α-1,2-mannosidase	Y
NRRL3_09843	uncharacterized	FAD-binding domain-containing protein	Y
NRRL3_08399	bgtB	glycoside hydrolase family 17 protein	M
NRRL3_00771	cwpA	cell wall mannoprotein	Y
NRRL3_08406	protF	serine carboxypeptidase, family S10*	Y
NRRL3_07375	uncharacterized	hypothetical protein	Y
NRRL3_06352	uncharacterized	purine nucleoside permease	Y
NRRL3_08160	ecmA	receptor L-domain-containing protein	Y
NRRL3_08703	uncharacterized	serine peptidase*	Y
NRRL3_01776	uncharacterized	sedolisin*	Y
NRRL3_09398	uncharacterized	β-1,3-glucanosyltransferase	Y
NRRL3_02860	uncharacterized	alkaline-phosphatase	Y

conclude that BGL and BXL encoding genes are controlled by XlnR and ClrA in *A. niger*. Furthermore, our proteomic results show that the main BGL (BglA) and BXL (XlnD/XynD) proteins are controlled by XlnR and ClrA, and XlnR appears to be able to compensate for the loss of *clrA* in the absence of *araR* and/or *clrB*. These results suggest that under our conditions, ClrA is not crucial for the regulation of BGL and BXL activities, but is part of the interactive regulatory system during growth of *A. niger* on wheat bran.

ClrB has been previously described to have a more extensive role in the breakdown of wheat straw compared with ClrA in *A. niger* (Raulo *et al.*, 2016), in particular with respect to cellulose utilization. Our proteomics and enzyme assay data suggest that this transcription factor controls the expression of cellobiohydrolases and endoglucanases, but not BGL genes, which is supported by the presence of a putative ClrB binding site (Li *et al.*, 2016) in the promoters of endoglucanases and cellobiohydrolases, but not in the analysed BGL gene (*bglA*). The impaired cellobiohydrolase and endoglucanase production shown by the *clrB* deletion mutant correlates with its inability to grow on cellulose.

The growth profile, enzyme activity assays and exoproteomics of the single Δ*araR* mutant suggest a minor role for AraR in the degradation of wheat bran. Deletion of *araR* only abolished growth on xyloglucan, which is a minor component of wheat bran. The abolished growth can possibly be explained by the reduced ability of Δ*araR* to remove L-arabinose units from the sidechains decorating xyloglucan, most likely mediated by AbfB (de Vries and Visser, 2001). This observation correlates with our results, showing highly reduced ABF activity, as well as reduced abundance of both analysed ABFs (AbfA and AbfB) in the exoproteome of Δ*araR* strain. All the genes encoding these proteins have been previously described to be under the control of AraR (Battaglia *et al.*, 2011).

In general, results from growth profiling and enzyme assays show that gene co-regulation is required for efficient utilization of crude substrates. Gene co-regulation by three different transcription factors (AraR, GaaR, RhaR) has already been reported in the case of sugar beet pectin degradation in *A. niger* (Kowalczyk *et al.*,

2017b). In our case, none of the single (hemi-)cellulolytic transcription factor deletion mutants showed strong reduction in growth on wheat bran, but a small reduction was observed in the quadruple mutant, indicating the integrative roles of the studied transcription factors in the overall utilization of this crude substrate. In addition, alternative carbon components of wheat bran can still largely compensate for the inability to use (hemi-)cellulose, as evidenced by the abundant growth of the quadruple mutant on wheat bran. The high abundance of CAZymes involved in starch utilization in the quadruple mutant's exoproteome suggested that utilization of starch was responsible for growth. Degradation of starch is mainly controlled by AmyR (Gomi *et al.*, 2000). The strong reduction in growth of the quintuple mutant indicated that starch is responsible for the only small growth reduction in the quadruple mutant, a phenotype confirmed by the exoproteome data of the quintuple mutant, where the major amylolytic enzymes (GlaA and AamA) were strongly reduced. Moreover, the deletion of *amyR* also resulted in the decreased abundance of the arabinoxylan arabinofuranohydrolase AxhA and the exo-inulinase InuE in the quintuple mutant. These results suggest that the role of AmyR extends beyond starch degradation, as it was also shown to control the expression of BGL, AGL and LAC encoding genes (vanKuyk *et al.*, 2012). However, the quintuple mutant still showed residual growth on wheat bran, most likely by utilizing proteins and other minor components, such as lipids (Morrison, 1978; Benoit *et al.*, 2015). The presence of these components is supported by the slightly upregulated protease profile of the quintuple mutant and the presence of lipases in the exoproteome. The most abundant proteases found in the exoproteome are controlled by PrtT (Punt *et al.*, 2008; Huang *et al.*, 2020). The major PrtT-controlled proteases (PepA, ProtA and ProtB) represented a relatively higher proportion of the exoproteome in the quintuple mutant compared with the quadruple deletion strain. This may be caused by the deletion of AmyR, as AmyR has been shown to have a negative effect on PrtT-mediated regulation of protease gene expression (Huang *et al.*, 2020). However, it is more likely that this is a starvation-induced response as utilization of the major carbohydrates is blocked in the quintuple

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Fig. 4. Analysis of non-CAZyme proteins produced by *A. niger* control and deletion mutant strains.
A. Relative composition of the total exoproteome of *A. niger* control and mutant strain supernatant samples originated from 3% wheat bran after 24 h. CAZymes are classified based on the substrates they are acting on and are indicated by different colours. Percentage values represent the abundance of non-CAZymes indicated by grey colour. The relative amount of proteins produced by each culture indicated on top has been determined by RCDC kit assay after protein precipitation. The represented samples originated from two independent proteomic analyses (Data S2B and S3B) indicated by different background colours.
B. Hierarchical clustering of non-CAZymes found in the supernatant samples of *A. niger* control and mutant strains. The colour code represents the averaged percentage value of the total exoproteome of duplicates. Proteins under putative control of PrtT are indicated by (*). Y = predicted secretion; M = putative secretion. Proteins with abundance lower than 0.1% of the total proteome in each sample were excluded from the analysis. The control, Δ*xlnR*, Δ*araR*, Δ*clrA*, Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* represent the results from the first proteomic data set (Data S2A), while the Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* data originate from the second proteomic data set (Data S3A).

mutant and starvation has been shown to cause protease induction (Nitsche *et al.*, 2012). The adaptation to the utilization of proteins in the quintuple mutant could be further investigated by the additional deletion of PrtT. This approach could possibly result in the abolishment of the residual growth that we observed on wheat bran. Finally, our data also show that while the quintuple mutant grows slower, it is apparently still highly viable as cell lysis did not occur extensively in this mutant, as indicated by a low total abundance of putative intracellular proteins. This supports our hypothesis that *A. niger* is able to utilize proteins and other minor non-carbohydrate components present in wheat bran when the utilization of every major and even residual carbohydrates is blocked in this species.

To our knowledge, this is the first study in which the major transcription factors involved in the regulation of CAZymes required for the utilization of the major (and most abundant) polysaccharides found in a crude substrate have been studied in combination in a fungus. Using combinatorial deletions, we achieved a strain showing a minimal CAZyme content in its extracellular proteome, making it unlikely for the fungus to be able to utilize any carbohydrates found in wheat bran. By analysing the single deletion mutants, we observed an unexpected growth improvement on wheat bran for the *clrB* deletion mutant, which correlated with an increased abundance of the main xylanolytic enzymes and improved BXL activity. This may be the result of a (in)direct interaction with XlnR, through a currently unknown mechanism. Moreover, we observed a transient growth of Δ*clrB* and an abolished growth of Δ*araR* mutant on xyloglucan. The deficient growth may correlate with the overall decreased abundance of xyloglucanases and α-L-arabinofuranosidases in the Δ*clrB* and Δ*araR* strains, respectively. These results provide leads for additional studies into the interaction between individual transcription factors.

In conclusion, our study shows hierarchical roles of the studied transcription factors with respect to (hemi-)cellulose utilization in wheat bran. XlnR is the major transcription factor for this substrate in *A. niger*, followed by ClrB, while ClrA and AraR show lower contribution (Fig. 5A). The apparent minor role of AraR, ClrA and ClrB in wheat bran utilization is most likely due to their overall low contribution to (arabino)xylan degradation. We also conclude that AmyR contributes to the degradation of wheat bran components (Fig. 5B). The hierarchy of all these transcriptional activators with respect to their relevance for wheat bran degradation may be species dependent and therefore explain the diverse enzyme sets published for different species during growth on wheat bran or other plant biomass substrates (Benoit *et al.*, 2015; de Vries *et al.*, 2017). This hypothesis is supported by the different roles of ClrA and ClrB in *N. crassa* (Coradetti *et al.*, 2012), *A. nidulans* (Coradetti *et al.*, 2012) and *A. oryzae* (Ogawa *et al.*, 2013), compared to *A. niger*, as well as the highly diverse set of target genes of XlnR in different fungi (Klaubauf *et al.*, 2014). Finally, we show that *A. niger* prefers to utilize (arabino)xylan over cellulose, but it is also able to maintain growth through the utilization of residual polysaccharides or even proteins and other minor components when the utilization of the main polysaccharides is blocked. These results highlight that *A. niger* possesses a flexible regulatory system, facilitating the use of most of the components found in plant biomass, which is likely a major reason for its high suitability for industrial applications.

Experimental procedures

Strains, media and growth conditions

Escherichia coli DH5α was used for plasmid propagation and was grown in Luria-Bertani (LB) medium supplemented with 50 μg ml^{-1} ampicillin (Sigma-Aldrich). Fungal strains used in this study were derived from the *A. niger* CBS 138852 (*cspA1, pyrG*$^-$, *kusA::amdS*) strain (Meyer *et al.*, 2007). The generated mutants were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute under accession numbers indicated in Table S4. Fungal strains were grown at 30°C on *Aspergillus* Minimal Medium (MM) or Complete Medium (CM) (de Vries *et al.*, 2004) supplemented with 1% D-glucose and 1.22 g l^{-1} uridine (Sigma-Aldrich).

Growth profiles were performed using *Aspergillus* MM containing 25 mM D-glucose/D-xylose/L-arabinose/maltose (Sigma-Aldrich), 25 mM cellobiose (Acros Organics) or 1% cellulose/beechwood xylan/xyloglucan/starch/wheat bran. The wheat bran used in this study was washed to remove free monosaccharides and a large part of the soluble starch (Nyombaire, 2012). Washing was performed by autoclaving wheat bran at 5% concentration in demineralized Milli-Q water. After autoclaving, the medium was centrifuged at 1800 × *g* for 10 min. The supernatant was removed and the insoluble wheat bran pellet was resuspended in sterile demineralized Milli-Q water. The suspension was centrifuged again, and the final supernatant-free pellet was resuspended in MM with 1% final concentration for growth profile or 3% final concentration for liquid cultures. All media were supplemented with 1.22 g l^{-1} uridine. All growth profile plates were inoculated in duplicates with 1000 spores and incubated at 30°C for up to 14 days. Pictures were taken after 5, 6, 8, 10 and 14 days of incubation and evaluated by visual inspection, taking into account colony diameter, mycelial density and sporulation.
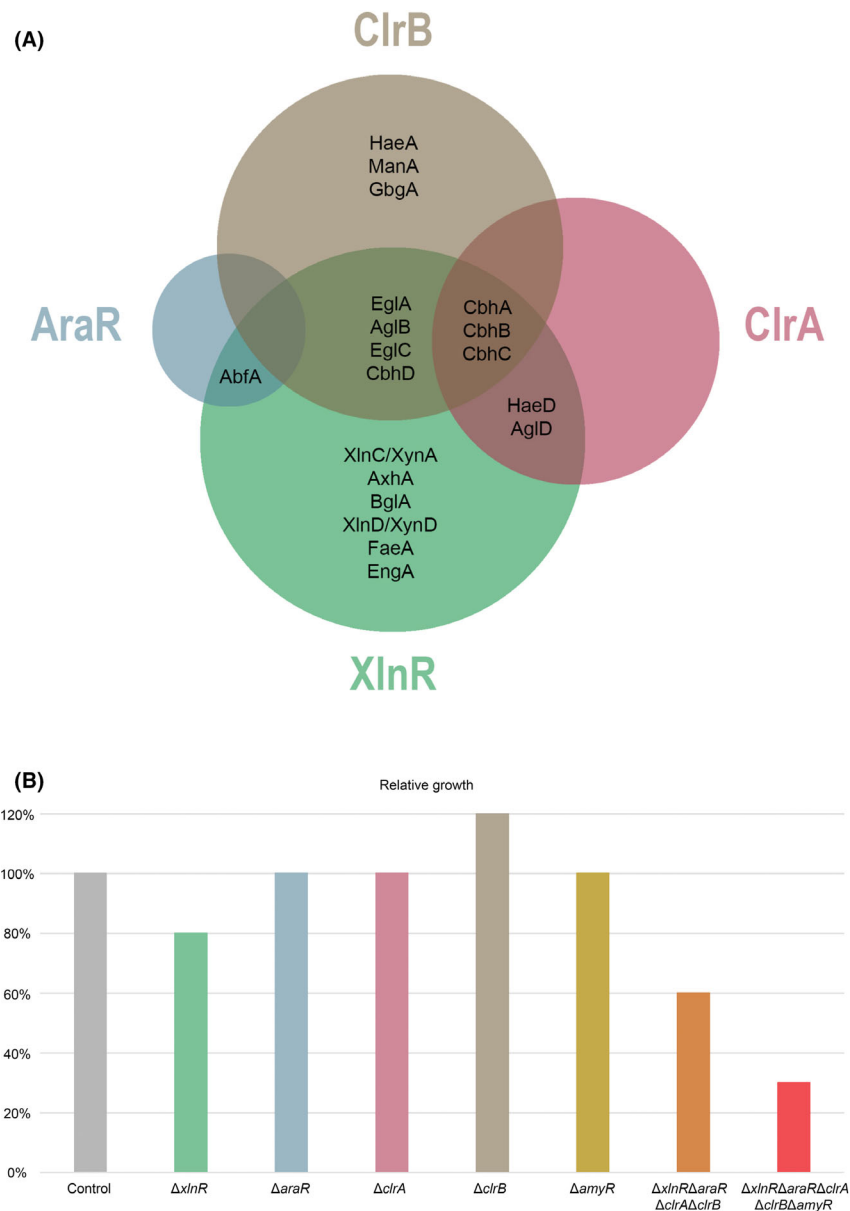
(A)

(B)

Fig. 5. Hierarchy of transcriptional activators involved in wheat bran utilization.

A. Contribution of XlnR, AraR, ClrA and ClrB in the regulation of major (hemi-)cellulases when grown on wheat bran. The (hemi-)cellulose-specific CAZymes that showed >two fold decrease in abundance in the exoproteome of single deletion mutants (Data S2A) are indicated under the control of the corresponding transcription factor. The regulated enzymes include one α-ʟ-arabinofuranosidase (AbfA), one β-ᴅ-arabinoxylan arabinofuranohydrolase (AxhA), two acetyl esterases (HaeA and HaeD), one feruloyl esterase (FaeA), two α-1,4-galactosidases (AglB and AglD), one β-1,4-endo-mannanase (ManA), one β-1,6-endoglucanase (EngA), one β-1,4-endoglucanase (EglC), four cellobiohydrolases (CbhA, CbhB, CbhC and CbhD), one β-1,4-glucosidase (BglA), one xyloglucanase (EglA), one β-1,4-endo-xylanase (XlnC/XynA) and two β-1,4-xylosidases (XlnD/XynD and GbgA).

B. Relative contribution of XlnR, AraR, ClrA, ClrB and AmyR towards utilization of wheat bran. Contribution of each transcription factor is represented by the relative growth reduction in the corresponding deletion mutants compared with the control. Relative growth has been estimated after 6 days of incubation at 30°C. No growth difference was observed between biological replicates.

For liquid cultures, freshly harvested spores were pregrown in 250 ml CM containing 2% ᴅ-fructose and 1.22 g l⁻¹ uridine for 16 h at 30°C in a rotary shaker at 250 rpm. After 16 h, mycelia were harvested by filtration through sterile cheesecloth, rinsed with MM, and approximately 2.5 g (wet weight) of mycelium were transferred into 50 ml MM containing 3% wheat bran. Supernatant samples were taken after 24 h of incubation at 30°C in a rotary shaker at 250 rpm. The samples were centrifuged (20 min, 3220 × *g*, 4°C), and cell-free

supernatant samples were stored at $-20°C$ until further processing.

Construction of mutant strains

The ANEp8-Cas9-*pyrG* plasmid (Song *et al.*, 2018), which contains the autonomous fungal replicating element AMA1 (Gems *et al.*, 1991), *pyrG* as selection marker, *cas9* gene and the guide RNA (gRNA) expression construct under the control of the proline transfer ribonucleic acid (tRNA^{Pro1}) promoter, was used in this study.

Selection of guide RNA (gRNA) sequences was performed using the GENEIOUS 11.1.4 software (https://www.geneious.com) based on the methodology described by Doench and collaborators (Doench *et al.*, 2014). Repair templates, which include the 5′ and 3′ flanking regions of the target genes, were amplified and fused together using fusion-PCR. Flanking regions represent 500–1000 bp homologous sequences before and after the target gene's open reading frame (ORF).

CRISPR/Cas9 plasmid construction, generation of *A. niger* protoplasts, transformation and purification of putative mutant strains was performed as previously described (Kun *et al.*, 2020). The Δ*xlnR*, Δ*araR*, Δ*xlnR*Δ*araR*, as well as the Δ*clrA*, Δ*clrB* and Δ*clrA*Δ*clrB* mutants were obtained by simultaneous double deletions using the *A. niger* CBS 138852 strain as background. The Δ*xlnR*, Δ*araR* and Δ*xlnR*Δ*araR* mutant strains have been used as background for further deletion of Δ*clrA* and Δ*clrB*, resulting in all possible combinations of deletions. Finally, the *amyR* gene was deleted in the Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* strain by performing a single deletion.

Mutant strains have been confirmed by analytical PCR, through the amplification of the target gene region. All primers used in this study were ordered from Integrated DNA Technologies (IDT, Leuven, Belgium) and are shown in Table S5.

SDS-PAGE and enzyme activity assays

Cell-free supernatant samples of 3% wheat bran liquid cultures were harvested after 24 h of incubation at 30°C in a rotary shaker at 250 rpm. Twelve microlitres of supernatant samples have been mixed with 4 μl loading buffer (10% of 1 M Tris-HCl, pH 6.8; 42% glycerol, 4% (w/v) SDS; 0.02% (w/v) bromophenol blue; 4% of 14.7 M mercaptoethanol), of which 10 μl aliquots have been analysed by SDS-PAGE as previously described (Kun *et al.*, 2020). Enzyme activities were evaluated based on colorimetric *p*-nitrophenol (pNP) assays. Supernatant samples (10 μl) were mixed with 10 μl 0.1% 4-nitrophenyl β-D-glucopyranoside (for BGL activity), 0.1% 4-nitrophenyl β-D-xylopyranoside (for BXL

activity), 0.1% 4-nitrophenyl α-L-arabinofuranoside (for ABF activity), 0.1% 4-nitrophenyl α-D-galactopyranoside (for AGL activity), 0.1% 4-nitrophenyl β-D-galactopyranoside (for LAC activity), 0.1% 4-nitrophenyl α-D-glucopyranoside (for AGD activity) or 0.1% 4-nitrophenyl maltoside (for GLA activity) substrates, 50 μl 50 mM NaAc (pH 5) and 30 μl demineralized water in a final volume of 100 μl. BGL, BXL and LAC activities were measured after 1 h, ABF activity was measured after 30 min, and AGL activity was measured after 15 min, while AGD and GLA activities were measured after 20 h of incubation at 30°C. The reactions were stopped by the addition of 100 μl of 0.25 M Na_2CO_3, and absorption values were measured at 405 nm wavelength using FLUOstar OPTIMA (BMG Labtech). All measurements were performed by using biological duplicates and technical triplicates.

Statistical analysis

The number of experimental replicates is described in the figure legends. Differences in enzyme activities were determined using the one-way analysis of variance (ANOVA) and Tukey's HSD test (Table S6). Statistical significance was referred for *p* value < 0.05. Analyses were done using STATGRAPHICS CENTURION XVI Version 16.1.17 (www.statgraphics.com/centurion-xvi).

Proteomic analysis

Proteins from 500 μl cell-free supernatant aliquots were precipitated by mixing them with two volumes of $-20°C$ methanol, followed by overnight incubation at $-20°C$. The protein solution was centrifuged at $20800 \times g$, 4°C for 20 min. The supernatant was aspirated, and the pellet was washed with 60% cold methanol solution and was resuspended in a 6 M urea, 100 mM ammonium bicarbonate pH 8 solution. Protein amounts have been determined colorimetrically by using the RCDC kit assay (Bio-Rad, Mississauga, ON, Canada). Five micrograms of protein samples of biological duplicates were digested with trypsin for proteomic analysis as previously described (Budak *et al.*, 2014). Dried peptide digest samples were solubilized in a solution of 5% acetonitrile, 0.1% formic acid and 4 fmol $μl^{-1}$ of trypsin-digested bovine serum albumin (BSA) (Michrom, Auburn, CA) used as internal standard. Five microlitres were analysed by LC-MS/MS using an Easy-LC II Nano-HPLC system connected in-line with a Velos LTQ-Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA). LC-MS/MS data peptide and protein identification were done using the *A. niger* NRRL3 protein sequence databases. Protein identification and quantification were performed using the Proteome Discoverer 2.2 (Thermo Fisher) precursor ion quantitation workflow.

Normalized individual protein area values were expressed as a fold value of the protein area value determined for the BSA internal standard. The abundance of proteins has been analysed using percentage values of the total exoproteome.

Heat maps for proteome data visualization were generated using the 'gplots' package of ʀ software, with the default parameters: 'Complete-linkage clustering method and Euclidean distance'. Proteins with a lower abundance than 0.1% of the total proteome in each sample were excluded from the analysis.

Binding site analysis

Binding site analysis for the target transcription factors was performed using the RSAT online tool (Thomas-Chollier *et al.*, 2008) (http://rsat-tagc.univ-mrs.fr/rsat/dna-pattern_form.cgi). The 1000 bp length promoter sequences upstream of the coding regions of the analysed genes were obtained from the JGI MycoCosm database (https://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html). Binding sites were searched using the 'DNA Pattern Matching' algorithm, with the default parameters of 'search on both strands' and 'prevent overlapping matches'. The reported putative binding motifs 5′-GGCTAR-3′ (de Vries *et al.*, 2002) and 5′-CGGNTAAW-3′ (Ishikawa *et al.*, 2018) for XlnR, 5′-CGGDTAAW-3′ (Ishikawa *et al.*, 2018) for AraR, 5′-CGGN$_8$CCG-3′ (Li *et al.*, 2016) for ClrB and 5′-CGGN$_8$CGG-3′ (Petersen *et al.*, 1999) for AmyR were analysed in this study.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

Author contributions

R.S.K. performed the experiments, analysed data and wrote the original manuscript. S.G. contributed to data analysis and manuscript writing. M.D.F. performed the proteomic analysis. A.T. and R.P.dV. designed the experiments, supervised the research, reviewed and edited the manuscript.

Data availability statement

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset identifier PXD023338 and 10.6019/PXD023338 (http://www.ebi.ac.uk/pride/archive/projects/PXD023338; Username: reviewer_pxd023338@ebi.ac.uk). All other data are available in the main text or in the supplementary files.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Enzymatic activities measured in this study. (A) Enzymatic activities and their substrates. The substrates present in wheat bran are highlighted in bold. (B) Enzyme assay results. Statistical analysis was performed using the converted (nmol/min/ml) values, while the visualization (Fig. 1C) was performed using the normalized mean and standard deviation (SD) values. (C) α-glucosidase (AGD) and glucoamylase (GLA) activity assay results. Statistical analysis was performed using the converted (nmol/min/ml) values, while the visualization (Fig. S3) was performed using the normalized mean and standard deviation (SD) values

Data S2. Extracellular proteome of *A. niger* control and Δ*xlnR*, Δ*araR*, Δ*clrA*, Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* mutant strains. (A) Proteomics results of *A. niger* control and Δ*xlnR*, Δ*araR*, Δ*clrA*, Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* mutant strains. Protein percentage values, which are < 0.1 are highlighted in red. Proteins, which show < 0.1% average value across all strains were excluded from analysis and are highlighted in grey. (B) Extracellular protein composition of control and mutant strains. Values represent the percentage of the total exoproteome.

Data S3. Extracellular proteome of *A. niger* control, Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* mutant strains. (A) Proteomics results of *A. niger* control, Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* mutant strains. Protein percentage values, which are < 0.1 are highlighted in red. Proteins, which show < 0.1% average value across all strains were excluded from analysis and are highlighted in grey. (B) Extracellular protein composition of control and mutant strains. Values represent the percentage of the total exoproteome.

Fig. S1. Relative contribution of XlnR, AraR, ClrA and ClrB towards utilization of wheat bran and related substrates. Contribution of each transcription factor is represented by the relative growth reduction of the corresponding single or multiple deletion mutants compared to the control. Relative growth has been estimated after 5, 6, 8, 10 and 14 days of incubation at 30°C. No growth difference was observed between biological replicates

Fig. S2. Relative contribution of AmyR towards utilization of maltose, starch and wheat bran. Contribution towards utilization of each substrate is represented by the relative growth reduction of the Δ*amyR* strain compared to the control, as well as the growth reduction of Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* compared to Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*.

Relative growth has been estimated after 6, 8 and 10 days of incubation at 30°C. No growth difference was observed between biological replicates

Fig. S3. α-glucosidase (AGD) and glucoamylase (GLA) activity of control, Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* mutant strains. Data represents the normalized mean values of biological duplicates and technical triplicates and the standard deviation. Letters (a-c) are shown to explain the statistical differences between samples within each specific enzyme assay. Samples showing different letters show significant differences among the strains within each specific enzyme assay (ANOVA and Tukey's HDS test, $P < 0.05$)

Table S1. Sugar composition of the wheat bran used in this study. The analysis was performed as described previously for other plant biomass substrates.

Table S2. Binding site analysis of analysed CAZymes. The position of the binding site is specified with respect to the transcription start codon. The orientation of binding sites is represented by F (forward strand) or R (reverse strand).

Table S3. Putative intracellular proteins found in WB liquid culture supernatants. Prediction of secretion was performed based on WoLF PSORT and Phobius protein localization and signal peptide prediction tools. Values represent the percentage of the total extracellular proteome. The proteins which were not detected in the samples are marked in grey cells.

Table S4. *Aspergillus niger* strains used in this study.

Table S5. Primers used in this study. Homology flanks are highlighted in red.

Table S6. Summary of the ANOVA analysis for each enzymatic assay.

Table S7. Enzyme abbreviations used in this study.