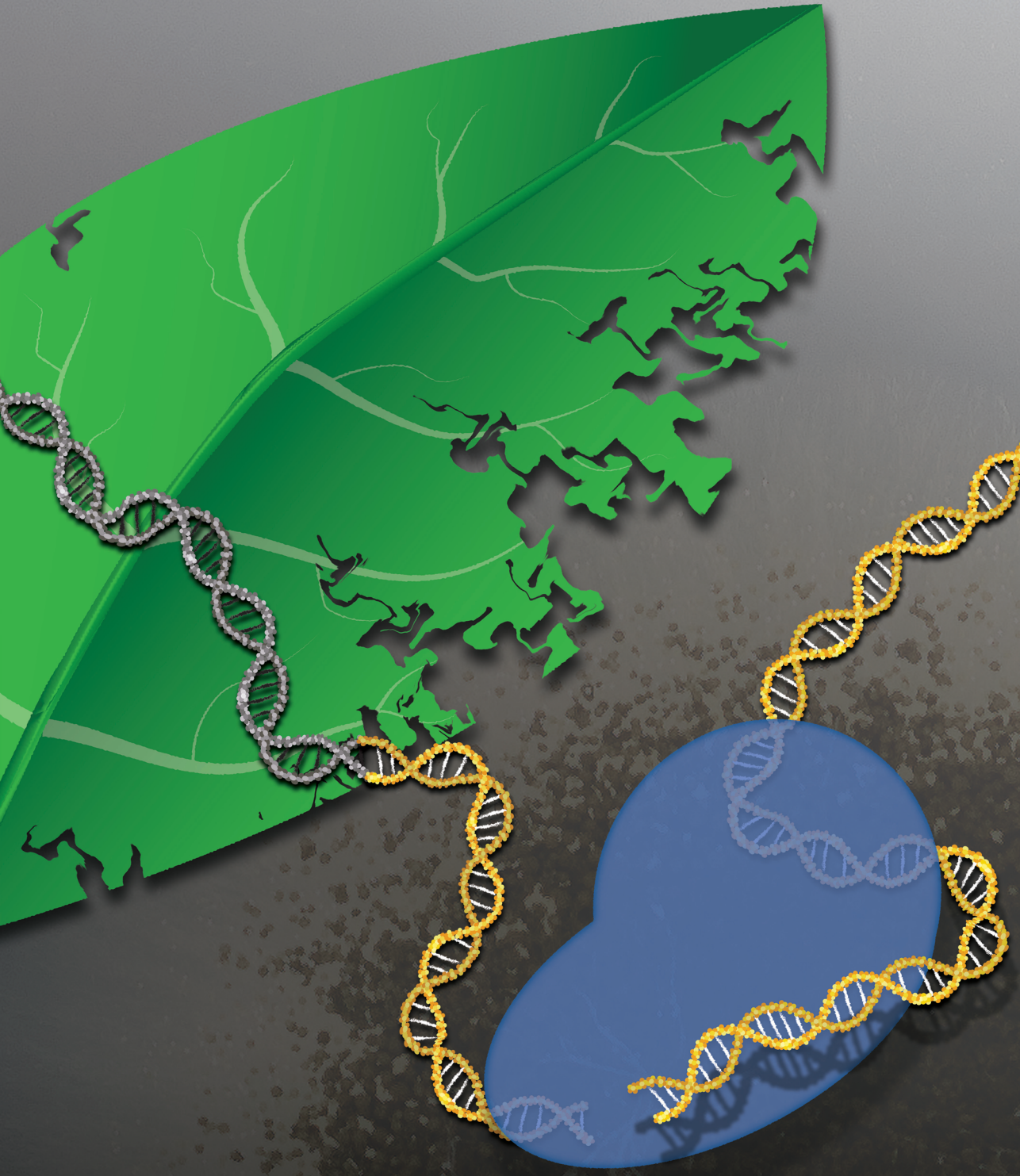

Harnessing the CRISPR/Cas9 system to study the regulatory network of plant biomass degradation in *Aspergillus niger*

Roland S. Kun

2022

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Harnessing the CRISPR/Cas9 system to study the regulatory network of plant biomass degradation in *Aspergillus niger*

Het toepassen van het CRISPR/Cas9-systeem om het regulatie netwerk van plantenbiomassa afbraak in *Aspergillus niger* te bestuderen

(met een samenvatting in het Nederlands)

A CRISPR/Cas9 rendszer alkalmazása az *Aspergillus niger* növényi biomassza lebontását irányító regulációs hálózatának tanulmányozására

(Magyar összefoglalóval)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op
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door

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geboren op 25 februari 1992
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To my wife, Dora

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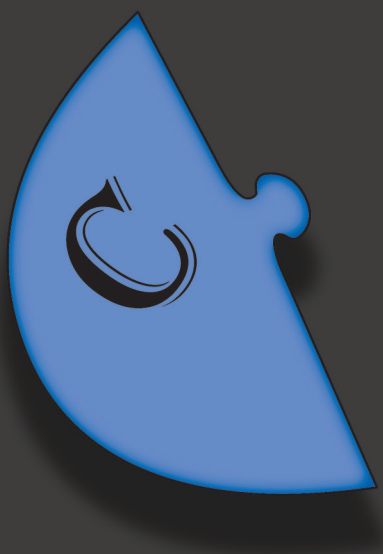

Chapter 1

General introduction

1. Filamentous fungi and their relevance

Filamentous fungi are a diverse group of eukaryotic organisms that are found in all biotopes on Earth. They have both positive and negative impacts on our society. On the one hand, fungal mycotoxins affect approximately 25% of the global crop output, leading to high economic losses worlwide[1]. Exposure to mycotoxins such as aflatoxin[2], can lead to severe health risks for animals and humans[3]. On the other hand, some filamentous fungi are important producers of industrially relevant metabolites, including several organic acids or drugs such as statins, antibiotics or immunosuppressives[4]. Moreover, a large number of saprobic filamentous fungi are highly efficient when it comes to degrading plant biomass, which is partly related to their high enzyme secreting capacities. Thus, they have a great ecological importance, as they are key players in the global carbon cycle[5].

Technological developments enabled the use of fungi in the industrial and agricultural biotechnology sectors. Industrial biotechnology, for example, has rapidly developed into a multibillion-dollar industry over the last century, in which fungi have played a crucial role. *Aspergillus niger* is one of the most iconic and revolutionary filamentous fungi in the biotechnology industry, firstly applied for the industrial production of citric acid[6–8]. However, *A. niger* is not the only filamentous fungus to offer such biotechnological opportunities, as other species, such as *Aspergillus oryzae*, have been used in various Japanese industrial fermentation processes for more than a millennium[9]. The *Aspergillus* genus has become one of the most important genera of filamentous fungi due to their broad diversity, their impact on societal needs, such as agriculture, industrial production, and basic research, and the relative ease of applicability in industrial processes[10]. On the other hand, several *Aspergillus* species, including *A. fumigatus*[11] and *A. flavus*[12], are pathogenic organisms, which can pose serious threats to human health. Nevertheless, several products from Aspergilli have “generally recognized as safe (GRAS)” status, allowing their use in food and feed applications[13]. Finally, a large number of *Aspergillus* genomes[10,14], including that of *A. niger*[15,16], became publicly available. This allows the scientific community to design and generate mutants with improved production abilities through targeted mutagenesis and/or rational design.

2. Plant biomass composition

Plant biomass is the most abundant carbon source on Earth, and it is mainly comprised of plant cell walls (Figure 1). Cell walls are composed of a network of interlinked

polysaccharides, including cellulose, hemicellulose and pectin. However, they also contain small amounts of protein and various amounts of lignin, an aromatic polymer that provides rigidity and endurance. Besides plant cell walls, plant biomass also includes storage polysaccharides, such as starch, inulin and gums[17].

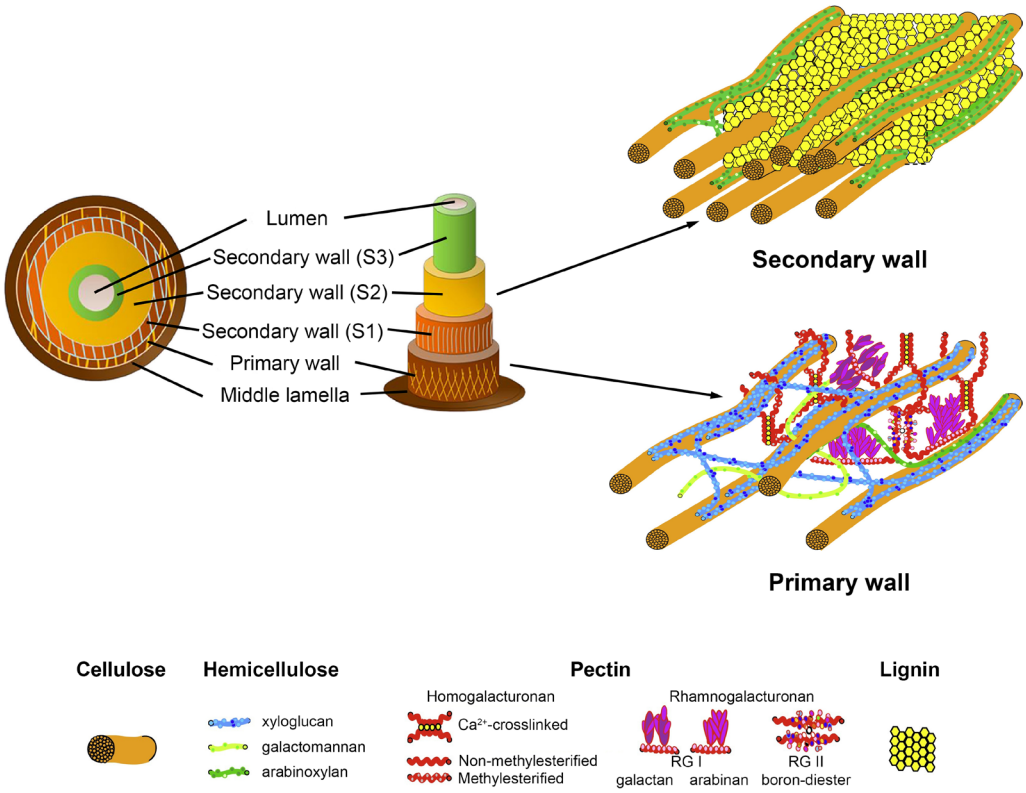

Figure 1. Schematic representation of plant cell walls. In general, plant cell walls contain three layers: middle lamella, primary cell wall and secondary cell walls (S1, S2 and S3). The primary cell wall is mainly composed of cellulose, hemicellulose and pectin, while the secondary walls also contain large amounts of lignin. Based on[18,19].

Cellulose is the most abundant plant cell wall polysaccharide. It is a linear homopolymer consisting of long chains of D-glucose units linked through β-1,4-glycosidic bonds[20]. These chains are tightly bundled into microfibrils, forming an insoluble fibrous material. Hemicelluloses are a more complex group of polysaccharides, often diversified due to interruption, branching, or decoration with different sugar monomers or oligomers along their structural backbone. This group of polysaccharides includes diverse structures with xylan (β-1,4-linked D-xylose), β-glucan (β-1,4-linked D-glucose) or mannan (β-1,4-

linked D-mannose) as backbone. Hemicelluloses are strongly associated with cellulose and play an important role in strengthening plant cell walls[21].

Pectins are the most complex polysaccharides found in plant cell walls. These are composed of homogalacturonan (a linear polymer of D-galacturonic acid units), xylogalacturonan (a D-xylose substituted homogalacturonan), rhamnogalacturonan I (RG-I) (consisting of an α-1,4-linked D-galacturonic acid and α-1,2-linked L-rhamnose backbone substituted with many structurally different side chains containing arabinosyl and galactosyl residues) or rhamnogalacturonan II (RG-II) (consisting of an α-1,4-linked D-galacturonic acid backbone with side chains composed of many different sugars, including D-galactose, L-arabinose, D-xylose and less common sugars, such as apiose)[22].

Starch is the most abundant form of storage polysaccharides in plants. It consists of an α-1,4-linked-D-glucose polymer (amylose) which can be branched through α-1,6-glycosidic linkages (amylopectin). Starch is typically present in seeds, roots and tubers of various plant species, such as potatoes, yams and cereals[23]. Another storage polysaccharide is inulin. It is composed of a branched β-2,1-linked chain of D-fructose units with a terminal D-glucose residue and is also typically present in roots and tubers of various plant species, including Jerusalem artichoke, chicory and dahlia[24–26].

3. Fungal enzymes and their applications

Plant polysaccharides cannot be directly taken up by fungal cells but need to be degraded to mono- or small oligosaccharides. Therefore, filamentous fungi produce numerous extracellular hydrolytic enzymes such as xylanases, mannanases, cellulases or polygalacturonases[17], as well as oxidative enzymes, including lytic polysaccharide monooxygenases (LPMOs)[27] to efficiently degrade lignocellulose. Table 1 presents the main enzymatic activities required for the degradation of major plant polysaccharides. These enzymes have been catalogued in the Carbohydrate Active enZyme (CAZy) database (www.cazy.org) in several families and subfamilies according to amino acid sequence similarity and enzymatic activities[28]. Most enzymes have a broad range of applications in diverse industrial fields, including biofuels and biochemicals, pulp and paper, textile, detergents or the food and feed industrial sectors.

The production of biofuels from degraded plant biomass has become an alternative for the rapidly depleting fossil fuels, thus contributing to a sustainable society. Bioethanol

and biomethanol can be produced from forest and agricultural residues. The degradation of lignocellulose for biofuel production requires the synergistic action of several cellulases (endoglucanases, cellobiohydrolases and β-glucosidases), hemicellulases (e.g., endoxylanases, β-xylosidases, endomannanases and β-mannosidases), oxidoreductases (e.g., laccases and LPMOs) or accessory enzymes, such as feruloyl esterases (FAEs)[5]. However, enzymes such as β-glucosidases represent a bottleneck in the bioethanol production as they are involved in the final enzymatic reaction of cellulose saccharification[29]. Therefore, many efforts are made to optimize cellulase production in several filamentous fungi, including *Trichoderma reesei*[30] or *Penicillium* sp[31]. With respect to biochemicals, FAEs are also used for the release of ferulic acid from lignin, which is an important chemical in the cosmetic and pharmaceutical industry[32]. Inulinases are also important for the generation of inulin-derived fructooligosaccharides and inulinooligosaccharides, which possess beneficial effects on human health[33].

In the pulp and paper industry, several plant polysaccharide degrading enzymes have been implemented as an environmentally friendly alternative of chemical pretreatment steps[34]. Mainly fungal xylanases[34], but also laccases, pectinases or α-amylases are used in this industrial sector[5]. The textile and detergent industries also use fungal enzymes for various applications. For instance, laccases can be used for bleaching fabrics while causing reduced environmental pollution[35], while thermo-active and alkaline lipases can improve the efficiency of detergents[36].

Fungal enzymes have a large application in the food and feed industrial sector, where they are applied in baking, juice clarification and in the production of jams, beverages or animal feed. Combinations of xylanases, glucanases, cellulases, pectinases, amylases, galactosidases, phytases, proteases and lipases are often used to improve the digestibility of grains in animal feed[5]. Pretreatment of animal feed with enzyme cocktails can result in higher nutritional uptake and net energy gain. For baking, xylanases, α-amylases, glucose oxidases and proteases are used to improve the rheological properties, consistency and shelf life of bread[5,37]. Pectinases are highly demanded for processes involved in juice clarification[38], preparation of jams[39] and wine production[40], while amylases, proteases, peptidases, xylanases and β-glucanases are the most important enzymes for malting and beer brewing[5]. Finally, it was shown that fungal mannanases efficiently degrade coffee mannan, resulting in improved concentration of coffee extracts[41], while pectinases, cellulases and hemicellulases are important for both coffee fermentation[42] and pretreatment of tea leaves and extracts[43].

Table 1. Fungal enzymes involved in plant biomass degradation.

Plant biomass polysaccharides		Fungal enzymes related to specific polysaccharides	
Group	**Polysaccharides**	**Abbreviation**	**Enzyme activity**
(Hemi-)cellulose	**Cellulose**	BGL	β-1,4-glucosidase
		CBH	cellobiohydrolase
		CDH	cellobiose dehydrogenase
		EGL	β-1,4-endo-glucanase
		LPMO	lytic polysaccharide monooxygenase
		ML-EGL	β-1,3/β-1,4-endoglucanase
	Xylan(s) (xylan, glucuronoxylan, arabinoglucuronoxylan, arabinoxylan)	ABF	α-L-arabinofuranosidase
		AGU	α-glucuronidase
		AXE	acetyl xylan esterase
		AXH	arabinoxylan arabinofuranohydrolase
		BGN	endo-1,6-β-D-glucanase
		BXL	β-1,4-xylosidase
		FAE	feruloyl esterase
		LPMO	lytic polysaccharide monooxygenase
		XBH	xylobiohydrolase
		XLN	β-1,4-endo-xylanase
	Mannan(s) (mannan, galactomannan, galactoglucomannan)	AGL	α-1,4-galactosidase
		BGL	β-1,4-glucosidase
		HAE	hemicellulose acetyl esterase
		LAC	β-1,4-galactosidase
		MAN	β-1,4-endo-mannanase
		MND	β-1,4-mannosidase
	Glucan(s) (xyloglucan, β-(1,3)(1,4)-glucan)	ABF	α-arabinofuranosidase
		AFC	α-L-fucosidase
		AGL	α-1,4-galactosidase
		AXL	α-xylosidase
		HAE	hemicellulose acetyl esterase
		LAC	β-1,4-galactosidase
		LPMO	lytic polysaccharide monooxygenase
		XEG/XG-EGL	xyloglucan endo-β-1,4-glucanase
		XGAE	xyloglucan acetyl esterase
Pectin	**Galacturonan(s)** (homogalacturonan, xylogalacturonan, apiogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II)	ABF	α-arabinofuranosidase
		ABN	endo-arabinanase
		BXL	β-1,4-xylosidase
		FAE	feruloyl esterase
		GAL	β-1,4-endo-galactanase
		GLN	exo-1,6-galactanase
		LAC	β-1,4-galactosidase
		PAE	pectin acetyl esterase
		PEL	pectin lyase
		PGA	endo-polygalacturonase
		PGX	exo-polygalacturonase
		PLY	pectate lyase
		PME	pectin methyl esterase
		RGAE	rhamnogalacturonan acetyl esterase
		RGL	rhamnogalacturonan lyase
		RGX	exo-rhamnogalacturonase
		RHA	α-rhamnosidase
		RHG	endo-rhamnogalacturonase
		UGH	unsaturated glucuronyl hydrolase
		URH	unsaturated rhamnogalacturonyl hydrolase
		XGH	xylogalacturonase
	Arabinan	ABF	α-arabinofuranosidase
		ABN	endo-arabinanase
		ABX	exo-arabinanase
		FAE	feruloyl esterase
	Galactan(s) (galactan, arabinogalactan)	EXG	exo-1,3-galactanase
		FAE	feruloyl esterase
		GAL	β-1,4-endo-galactanase
		LAC	β-1,4-galactosidase
Storage polysaccharide	**Starch** (amylose, amylopectin)	AGD	α-glucosidase
		AMG	amylo-α-1,6-glucosidase
		AMY	α-amylase
		GLA	glucoamylase
		LPMO	lytic polysaccharide monooxygenase
	Inulin (β-(1,2)-fructan)	INU	endo-inulunase
		INX	exo-inulinase
		SUC	invertase

4. Regulation of plant biomass degradation

In order to produce the enzymes required for the degradation of plant polysaccharides, the presence of polysaccharide derivatives, such as mono- and/or disaccharides is required[44]. The uptake of such mono- and disaccharides triggers signaling pathways that subsequently activate transcription factors (TFs), which can either activate or repress gene expression. The majority of fungal transcriptional activators belong to the Zn_2Cys_6 class proteins, which after activation are relocated into the cell nucleus. There they can bind to the promoter regions of their target genes and induce their expression to produce enzymes necessary for substrate degradation as well as utilization of the available carbon sources[44,45]. However, mono- and disaccharides do not typically occur as free sugars in plant biomass. It is still unclear how substrate degradation is induced in fungi. It has been hypothesized that certain "scouting" enzymes are induced under carbon-starvation conditions, which subsequently provide inducing molecules to fully activate a response to degrade the available carbon source[46,47]. However, it is more likely that fungi constitutively produce low levels of some CAZymes to subsequently initiate complete activation when an inducer concentration threshold is reached[45,48].

The three most abundant groups of plant cell wall polysaccharides, cellulose, hemicellulose and pectin as well as storage polysaccharides are prominent in plant biomass, for which plant biomass-degrading enzymes can be produced under the regulation of numerous interplaying TFs[49–51]. Understanding the transcriptional regulatory systems involved in plant biomass degradation among Aspergilli offers great potential for advancing biotechnology. The following sections will primarily focus on the current knowledge, including functionality and network interactions of several TFs in *Aspergillus* spp., which are involved in the degradation of plant biomass. Major transcriptional activators involved in the degradation of cellulose, hemicellulose, pectin and storage polysaccharides will be discussed separately.

Besides activators, transcriptional repressors also play a crucial role in the regulation of gene expression in order to maintain an energetically favorable balance. The main transcriptional repressor playing a major role in CCR is CreA[52]. Transcriptional repressors will be discussed in a separate section.

4.1 Transcriptional activators of plant biomass degradation

The degradation of (hemi-)cellulose is mainly regulated by the transcriptional activators

XlnR[53], ClrA[54] and ClrB/ManR[54]. AraR[55] is also involved in the regulation of hemicellulose degradation[56] and pentose catabolism[55], although it has a major role in the regulation of pectinolytic activities[50]. Thus, AraR will be partly discussed in both sections regarding hemicellulose and pectin degradation. Pectin degradation is additionally controlled by GaaR[57] and RhaR[58], with putative involvement of GalX[59]. Finally, AmyR[60] and InuR[61] play a crucial role in the degradation of the storage polysaccharides starch and inulin, respectively.

Besides these TFs, additional *Aspergillus* TFs or modulators have been described which show involvement in plant biomass degradation or sugar catabolism. These include the maltose regulator MalR, identified in *A. oryzae*[62], the cellobiose regulator ClbR, described in *Aspergillus aculeatus*[63], and the D-galactose regulator GalR[64] and MADS-box protein McmA[65], both described in *Aspergillus nidulans*. Additionally, several TFs involved in the regulation of plant biomass degradation have been described in Sordariomycetes. These include: the modulator of glucose sensing and carbon catabolite repression (CCR) VIB1[66], described in *Neurospora crassa*, which shows a positive effect on the expression of hydrolytic enzyme genes and the expression of *clr-2* (ortholog of *clrB*/*manR*); the (hemi-)cellulolytic activators ACE2[67] and ACE3[68], both identified in *T. reesei*; and the L-arabinose responsive TF, ARA1, described in *Pyricularia oryzae* (*Magnaporthe oryzae*)[69] and *T. reesei*[70], which is the functional analog of AraR in Eurotiomycetes.

4.1.1 Transcription factors involved in (hemi-)cellulose degradation

In Aspergilli, there are three primary TFs that govern the gene regulation for (hemi-) cellulose degradation, namely XlnR[53], ClrA and ClrB/ManR[71,72]. ClrA and ClrB are orthologs of CLR-1 and CLR-2, which were originally described in *N. crassa*[54]. These TFs are required for utilization of cellulose in *N. crassa*, but not for hemicellulase activity on xylan[54]. Moreover, their orthologs have already been (partially) characterized in several *Aspergillus* spp., including *A. nidulans* (ClrB)[54], *A. oryzae* (ManR)[72] and *A. niger* (ClrB)[71]. It should be noted that the ClrB equivalent in *A. oryzae* is known as ManR, due to its initial association with enzyme regulation for mannan-degradation[72]. A subsequent study also showed the involvement of *N. crassa* CLR-2 in the regulation of mannan utilization[73], but the regulation of mannanolytic activities is not fully conserved in the case of *A. nidulans* ClrB[74]. In *N. crassa*, CLR-2 is a major activator of cellulase gene expression and is under the transcriptional regulation of CLR-1, which acts as a crucial cellulose-sensor[54]. Conversely, ClrA was shown to be less prominent as a cellulose-

sensor in *A. nidulans*[54]. However, utilization of cellobiose, a degradation product of cellulose, was defective upon deletion of ClrA, suggesting that ClrA is still necessary for optimal cellulolytic activity[54]. These findings strongly support that ClrA may have a conserved role in cellobiose-sensing pathways among filamentous ascomycetes, albeit to different extent between species[75]. On the other hand, CLR-2 and ClrB were shown to be highly conserved in both *N. crassa* and *A. nidulans,* respectively, as essential cellulase activators[54]. As aforementioned, these TFs have also been characterized to some extent in other *Aspergillus* spp., such as *A. niger* and *A. oryzae*, where ClrB and ManR were both shown to be crucial for the regulation of cellulolytic activities[71,72]. Additionally, it was shown that the role of ClrB in the regulation of cellulase gene expression is also conserved in *Penicillium oxalicum*[76,77], suggesting a conservation of a similar regulatory mechanism for cellulose utilization between Aspergilli and Penicillia.

Although it has been reported that orthologs of CLR-1/ClrA and/or CLR-2/ClrB are present in genomes of diverse filamentous ascomycetes[54], these are still poorly studied in several species and further studies are required for the validation of their functions. Apart from CLR-1/ClrA and CLR-2/ClrB, two additional (in)direct modulators of the cellulolytic system were described in *N. crassa*, CLR-3[78] and CLR-4[79]. CLR-3 was associated with the repression of *clr-1* in the absence of an inducer[78], while CLR-4 was shown to regulate the expression of the three major (hemi-)cellulolytic TF genes *clr-1*, *clr-2* and *xlr-1* and key genes involved in the cyclic adenosine monophosphate (cAMP) signaling pathway in *N. crassa*[79]. An ortholog of CLR-4 was also identified in another Sordariomycete fungus, *Myceliophthora thermophila*, showing a comparable role to its *N. crassa* ortholog[79]. However, it remains to be seen if functional analogs of CLR-3 and CLR-4 are present in *Aspergillus* spp.

XlnR/Xyr1 was the first TF identified to be involved in the regulation of xylanase gene expression in filamentous fungi[80], and in *A. niger,* XlnR later became one of the most studied TF involved in glycoside hydrolase gene regulation in filamentous fungi. Studies on XlnR and its homologs have also expanded into other reference or industrial *Aspergillus* species, such as *A. nidulans*[81] or *A. oryzae*[82]. XlnR orthologs have also been described in other Aspergilli such as *Aspergillus fumigatus*[83] and *Aspergillus tamari*[84]. Additionally, XlnR was found to be conserved in nearly all studied filamentous ascomycetes[85], including *P. oxalicum*[76], *Fusarium oxysporum*[86], *T. reesei* (Xyr1)[87] and *Fusarium graminearum* (Xyr1)[88].

In *A. niger*, it was demonstrated that XlnR is inactive in the cytoplasm and undergoes nuclear import upon D-xylose induction, where it can function as a first step regulator

of CAZy and D-xylose catabolic pathway gene expression[89,90]. XlnR has been shown to primarily regulate the expression of several cellulases and hemicellulases, including xylanases, β-xylosidases, cellobiohydrolases, endoglucanases, galactosidases, arabinofuranosidases and carbohydrate esterases in *A. niger*[91]. The involvement of XlnR in the regulation of cellulase gene expression in *Aspergillus* spp.[53] suggests a diversity of cellulase regulation mechanisms among filamentous ascomycetes via XlnR, ClrA, ClrB and their homologs[75]. Additionally, one study in *A. niger* demonstrated that both *clrA* and *clrB* transcript levels decreased significantly after deletion of *xlnR*[71]. These findings suggest the possibility that XlnR directly regulates the expression of *clrA* and *clrB*.

In *Aspergillus*, AraR shows a strong sequence similarity to XlnR, and these similarities are speculated through the help of evolutionary analysis to be the product of a gene duplication event[55]. Studies in *A. nidulans* and *A. niger* demonstrated both gene co-regulation, as well as antagonistic interaction between AraR and XlnR[56,92–94]. For instance, AraR and XlnR are both crucial for the functionality of the pentose catabolic pathway (PCP), showing overlapping and synergistic roles in promoting the expression of several genes involved in this pathway[55,56]. Both D-xylose and L-arabinose are sugars often derived from hemicellulose, and are catabolized through this pathway. Additionally, both TFs were shown to regulate the expression of several genes involved in the oxidoreductive D-galactose catabolic pathway in *A. nidulans* and the pentose phosphate pathway in *A. niger*[81,94,95]. This form of co-regulation is not particularly surprising as D-galactose, L-arabinose and D-xylose are often present together in nature[94]. However, one study showed that the role of AraR differs significantly in the PCP between *A. nidulans* and *A. niger*, despite being taxonomically closely related[81]. Conversely, the same study showed that XlnR functionality is similar between *A. nidulans* and *A. niger*[81]. A BlastP analysis within the same study suggested the involvement of a currently unknown regulator in the regulation of PCP in *A. nidulans*[81]. Further variation within Eurotiomycetes was demonstrated in another study, where the single deletion of the *xlnR* homolog in *P. oxalicum* resulted in increased α-L-arabinofuranosidase production[96]. This increase was linked to AraR activity. However, similar outcomes were not observed in *A. niger*, which emphasizes the different regulatory mechanisms between these species, particularly regarding the role of AraR and its interplay with XlnR[56]. The significant overlap between the activity of XlnR and other transcriptional regulators shows that the regulation of substrate utilization is a highly interconnected and intricate process[56,71]. More research is needed to further elucidate the contribution of XlnR to other regulatory mechanisms.

4.1.2 Transcription factors involved in pectin degradation

Several TFs regulate genes involved in pectin degradation, including AraR[55], GaaR[57], RhaR[58] and possibly GalX[59], which has been shown to primarily play a role in D-galactose catabolism in *A. nidulans* and *A. niger*[59,64].

AraR is a well-known Zn_2Cys_6-type TF, which typically responds to the presence of L-arabinose or L-arabitol[97,98]. It is responsible for regulating the expression of a diverse set of CAZy genes in *Aspergillus* spp., such as *A. nidulans*[81], *A. niger*[55,56,81] or *A. oryzae*[93]. Despite the previously described similarities between XlnR and AraR, these TFs also show distinct aspects of functionality, as it has been demonstrated that AraR displays a more prominent contribution to the regulation of pectinolytic genes, including genes coding for α-L-arabinofuranosidases (*abfA, abfB*), β-galactosidases (*lacA*) and β-xylosidases (*gbgA*)[50]. For example, α-L-arabinofuranosidases play an important role in the degradation of pectin-containing substrates by removing L-arabinose units from the decorating side chains[50,99,100]. Moreover, it was shown that AraR orthologs are also present in other fungal taxa[55]. For example, an AraR ortholog has already been characterized in *P. oxalicum*[96,101]. In this study, it was demonstrated that AraR can be genetically manipulated to produce significantly increased amounts of α-L-arabinofuranosidases[96].

Several other TFs involved in pectin degradation can be found in diverse *Aspergillus* spp., all of which typically respond to certain monomers present in complex pectin structures. L-rhamnose can typically be found in the pectic polysaccharides RG-I and RG-II found in the plant cell walls. L-rhamnose has been shown to induce and upregulate the expression of several genes encoding α-L-rhamnosidases, endopolygalacturonases, exo-rhamnogalaturonan hydrolases and rhamnogalacturonan acetylesterases in filamentous fungi. These enzymes are required for the degradation of the previously mentioned pectic polysaccharides[58,102]. L-rhamnose-mediated induction of RhaR was shown in *A. nidulans*[102] and *A. niger*[58,103,104], which resulted in upregulated expression of enzymes for both the degradation of L-rhamnose-containing substrates as well as for L-rhamnose catabolism. More recently it was shown that the L-rhamnose derivative 2-keto-3-deoxy-L-rhamnonate (L-KDR) is responsible for the induction of the RhaR-responsive genes in *A. niger*[105]. Other *Aspergillus* species such as *A. aculeatus* have also been suggested to contain similar regulatory systems based on rhamnogalacturonan hydrolase RghA activity[106].

D-galacturonic acid is a prominent monomer in pectin structures. Its catabolic intermediate, 2-keto-3-deoxy-L-galactonate acts as the inducer of GaaR in *A. niger*[107].

Interestingly, both GaaR and RhaR inducers are 2-keto-3-deoxy-L derivatives, which may indicate a shared ancestral induction system. Induction of GaaR activity promotes upregulation of pectinase gene expression, such as exo-polygalacturonases, as well as genes involved in transport and catabolism of D-galacturonic acid[57]. Moreover, it has been shown in *A. niger* that overexpression of GaaR results in upregulation or constitutive expression of pectinases, putative D-galacturonic acid transporters, and enzymes involved in D-galacturonic acid catabolism, even in the absence of D-galacturonic acid[108]. In contrast, a knockout of GaaR results in downregulation of pectinases and genes involved in D-galacturonic acid utilization, highlighting the crucial role of GaaR for growth on D-galacturonic acid-rich substrates[57]. Based on a phylogenetic analysis, Alazi et al. also showed that 19 out of 20 different *Aspergillus* spp. contain a GaaR ortholog, being only absent in *Aspergillus glaucus*[57]. This also explains why *A. glaucus* lacks the ability to grow on D-galacturonic acid as a sole carbon source[57].

D-galactose is a very common monomer found in pectin and it was shown to be the primary inducer of GalX/GalR activity[64]. In *A nidulans*, these two Zn_2Cys_6 transcriptional activators were found to be involved in the regulation of D-galactose metabolism[64]. Moreover, it was shown that GalR activity may be under the control of GalX, and that XlnR and AraR are also involved in D-galactose catabolism in *A. nidulans*[94] and *A. niger* (under review). Interestingly, GalX can be found among various Aspergilli such as *A. niger*, *A. nidulans*, *A. oryzae*, *A. fumigatus* and *Aspergillus flavus*, while GalR was shown to be unique to *A. nidulans*[59] and other species of the section *Nidulantes*. Additionally, it was also shown that both GalX and GalR are absent in *Aspergillus terreus* and *Aspergillus clavatus*[59]. This likely suggests a significantly different catabolic system for D-galactose among such fungi that lack both GalR and GalX. Since D-galactose is a prominent monomer in pectin structures, it might suggest that GalX could also be involved in pectin degradation. However, currently there is no evidence, to our knowledge, indicating that GalX truly has a role in pectin degradation. Further investigation is required to clarify this.

Finally, research has also demonstrated strong interplay and combinatorial gene regulation between GaaR, AraR and RhaR[50], as well as synergistic activities of a large number of pectinolytic enzymes controlled by these TFs[109].

4.1.3 Transcription factors involved in storage polysaccharide degradation

Storage polysaccharides are a form of stored or reserved energy within a living organism, mainly represented by starch and inulin. AmyR and InuR are both highly conserved Zn_2Cys_6 TFs required for amylolytic and inulinolytic gene regulation among Aspergilli[60,61]. AmyR has been described to be present in several *Aspergillus* spp., namely *A. niger*[110], *A. oryzae*[111] and *A. nidulans*[112], but also within other taxa, including *P. oxalicum*[76]. AmyR is typically induced in the presence of starch or maltose and it upregulates the production of amylolytic enzymes such as α-amylases, glucoamylases and α-glucosidases for starch degradation[113]. In *A. nidulans,* isomaltose has been suggested to be the inducer for AmyR activity[114,115]. Additionally, D-glucose-mediated induction, as well as the regulation of α-/β-galactosidase activity was shown in *A. niger*[116], which indicates a broader role of AmyR in plant biomass degradation.

In *A. niger,* inulinolytic enzymes have been shown to be induced when either inulin or sucrose are present[117]. Several inulin-degrading enzymes have been identified in *A. niger*, including exo-inulinases[118], endo-inulinases[119] and invertases[120]. Results from another study suggest that *A. niger* utilizes inulin and sucrose by expressing various extracellular enzymes and sugar transporters, all of which are under transcriptional control of InuR[61]. Interestingly, phylogenetic analysis showed that InuR is closely related to AmyR[61]. Moreover, Yuan et al. have indicated that the proposed transcriptional binding site of InuR is very similar, if not identical, to that of AmyR, which was shown to be present in the promoters of several amylolytic genes in *A. niger*[61]. These strong similarities between InuR and AmyR indicate that they likely have arisen from a common ancestor, similar to XlnR and AraR. Interestingly, the same study showed that *A. niger* growth on inulin was not abolished when InuR was deleted[61]. Further growth reduction was observed when both InuR and AmyR were deleted, which suggests a correlation between these two TFs for inulin utilization[61].

4.2 Transcriptional repressors involved in plant biomass degradation

In order to maintain energetic balance, certain genes also need to be repressed when the encoded enzymes are not required for the degradation of carbon sources found in the environment. The most studied transcriptional repressor is CreA/CRE1, which has been described as a well conserved repressor in filamentous fungi[85,121–123].

CreA/CRE1 is a Cys_2His_2 class TF and it regulates CCR, affecting the expression of a broad range of CAZy genes[123–125]. However, it was shown that mutations of *creA/cre1* can also affect additional biological functions, including colony morphology[126], sporulation[127], carbon and nitrogen metabolism[128–130], as well as secondary metabolism[131].

Several additional repressors involved in the regulation of cellulose or hemicellulose gene expression have been described primarily in *T. reesei*. These include the cellulase repressors ACE1[132] and Rce1[133], the xylanase repressor XPP1[134] and the GH11 endoxylanase-specific repressor SxlR[135]. Similarly, a hemicellulose repressor, HCR-1, has also been described in *N. crassa*[136]. Moreover, in the same fungus, CLR-3 has been identified as a repressor of cellulolytic activities through its interaction with CLR-1[78]. Besides (hemi-)cellulolytic repressors, GaaX has been associated with the repression of GaaR activity in the absence of D-galacturonic acid in *A. niger*[137]. Moreover, it has been suggested that a GaaR-GaaX activator-repressor module is well conserved among ascomycete fungi[137].

Although the aforementioned transcriptional repressors belong to the Cys_2His_2 class proteins (ACE1, HCR-1 and CreA/CRE1), Rce1 and SxlR, both identified in *T. reesei*, belong to the Zn_2Cys_6 class proteins. Despite the general association of fungal transcriptional activators with Zn_2Cys_6 class proteins and repressors with Cys_2His_2 class proteins, the Cys_2His_2 Rxe1 TF described in *T. reesei* was shown to positively affect the expression of the (hemi-)cellulolytic regulator gene *xyr1* by direct binding to its promoter[138]. These results represent the diversity of TFs taking part in gene activation or repression in filamentous fungi.

5. Aims and outline of this thesis

The availability of a large number of fungal genomes has led to significant progress in the identification and characterization of fungal TFs. The identification of the regulons and interactions of several TFs allowed the generation of fungal strains with improved enzyme cocktails for efficient plant biomass degradation[139]. Nevertheless, there is still much to be learned about the complexity of the fungal regulatory network for further optimization of enzyme production. The establishment of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas) gene editing system in filamentous fungi[140–142] has revolutionized strain engineering by allowing the implementation of multiplex gene editing or precise sequence modifications.

The aim of this thesis was to apply the CRISPR/Cas9 gene editing system in the filamentous fungus *A. niger* to generate various mutations (gene deletion, point mutation, domain swapping) of TFs involved in the regulation of plant biomass degradation, to get a deeper insight into the regulatory network controlling this process. The CRISPR/Cas9 system was used to generate a large number of single and combinatorial deletion strains, as well as mutants carrying precise on-site modifications in selected native genes. Results described in this thesis provide more insight into the possibilities of strain engineering using CRISPR/Cas9 in *A. niger*, and better understanding of the fungal regulatory network governing the degradation of agricultural crude substrates.

In **Chapter 2**, different approaches of strain engineering for improved production of fungal enzyme cocktails have been reviewed. This chapter provides a summary of various applications of classical transformation methods, as well as the establishment and application of novel gene editing methods, such as CRISPR/Cas9, in different fungal species. Additionally, this chapter provides an insight into the omics technologies and their implementation into fungal strain engineering.

Chapter 3 presents a method for rapid generation of constitutively active forms of two TFs, XlnR and GaaR. The CRISPR/Cas9 system was used to introduce on-site mutations in the endogenous *xlnR* and *gaaR* sequences, using short single stranded oligonucleotides as repair templates. The generated mutants showed increased production of enzymes required for the release of D-xylose, L-arabinose or D-galacturonic acid, which resulted in improved saccharification of wheat bran or sugar beet pulp.

Additionally, the CRISPR/Cas9 system allowed us to replace the N-terminal region of the endogenous *xlnR* gene with the N-terminal region of *gaaR*, resulting in a chimeric GaaR-XlnR TF mutant. This mutant showed the activation of pectinolytic functions in the presence of D-xylose. The alteration of TF specificity is a promising tool for the generation of mutants which can produce high value enzyme cocktails on cheap agricultural substrates. The results of this study are presented in **Chapter 4**.

The CRISPR/Cas9 system was also used for the generation of a large number of single and combinatorial TF deletion strains. By analyzing these deletion mutants, it is possible to assess which genes are under the control of a specific TF and which genes are co-regulated by multiple TFs. One study involved the analysis of an *A. niger* Δ*clrB* mutant. Our results provide more information about the inducing condition and the regulon of this TF. These results are described in **Chapter 5**.

In **Chapter 6**, the generation and analysis of single and double deletion mutants of *amyR* and *inuR* is presented, encoding the regulators involved in storage polysaccharide degradation. This study shows a culture condition-dependent involvement of AmyR in the utilization of sucrose and inulin, which was mainly evidenced by data originated from solid plate cultures.

In **Chapter 7**, we report the generation of a complete set of XlnR-AraR-ClrA-ClrB deletion mutants through consecutive gene deletions by CRISPR/Cas9. We evaluated the role of these four TFs with regard to wheat bran utilization, with main focus on (hemi-)cellulose degradation. Moreover, the generation and analysis of a multi-deletion stain which included the additional deletion of *amyR*, showed the importance of starch utilization in wheat bran. This study provides a better understanding of the regulatory network governing wheat bran degradation.

Chapter 8 describes the regulation of sugar beet pulp degradation by *A. niger*. A large set of single and combinatorial deletion mutants were generated using CRISPR/Cas9. The deleted genes included four TF genes with putative involvement in pectin degradation (*gaaR, rhaR, araR,* and *galX*) and two TF genes with involvement in (hemi-)cellulose degradation (*xlnR* and *clrB*). Transcriptome analysis of the control and multi-deletion strains revealed the adaptation of *A. niger* towards the utilization of sugar beet pulp components during the experimental time course.

Finally, the results of this thesis are summarized and discussed in **Chapter 9**.

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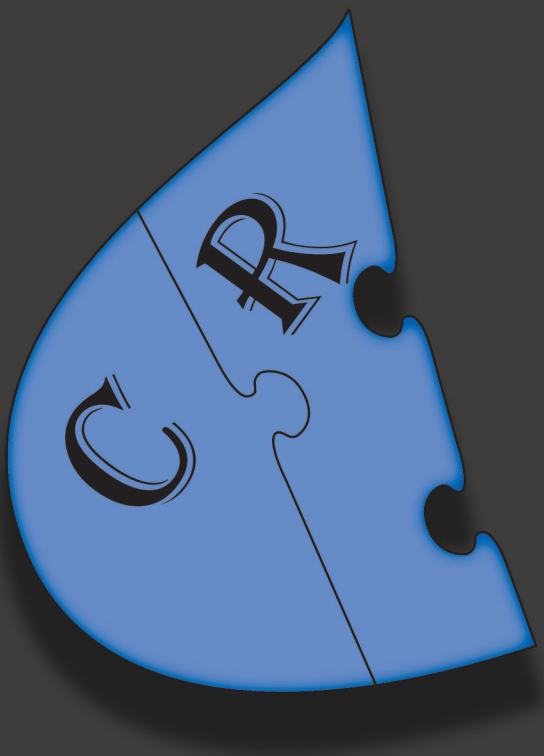
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Chapter 2

Developments and opportunities in fungal strain engineering for the production of novel enzymes and enzyme cocktails for plant biomass degradation

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Abstract

Fungal strain engineering is commonly used in many areas of biotechnology, including the production of plant biomass degrading enzymes. Its aim varies from the production of specific enzymes to overall increased enzyme production levels and modification of the composition of the enzyme set that is produced by the fungus. Strain engineering involves a diverse range of methodologies, including classical mutagenesis, genetic engineering and genome editing. In this review, the main approaches for strain engineering of filamentous fungi in the field of plant biomass degradation will be discussed, including recent and not yet implemented methods, such as CRISPR/Cas9 genome editing and adaptive evolution.

1. Introduction

Filamentous fungi are a diverse group of eukaryotic organisms, which have both positive and negative impacts on our society. Some of them are responsible for the contamination of food and fodder and infection of living organisms[1]. On the other hand, some filamentous fungi are potent producers of industrially relevant metabolites such as antibiotics, statins or immunosuppressive drugs as well as organic acids. Filamentous fungi have a central role in the production of various biotechnologically important enzymes, such as those degrading complex plant materials[2,3]. These fungal enzymes are used e.g. in the pulp and paper, food and feed, and textile and detergent industry. In addition, more recent application of fungal enzymes is in the biofuels and biochemicals industries[4].

Due to the broad range of applications fungal enzymes are used for, the production level of the enzymes and the composition of the enzyme sets produced have been actively studied over the last decades, and have been the target of strain engineering in many academic and industrial studies[5–8]. Increasing enzyme production level in general is important for many applications, as it will directly affect the overall process costs. However, also ensuring that the right set of enzymes is produced, including all required activities and avoiding unwanted activities, is highly relevant. Often wild type strains do not produce the protein at levels required in industry, nor do they produce exactly the desired enzymes set. To solve this problem, strain engineering has generated industrial fungal strains that have superior performance compared to wild type strains. Genetic engineering approaches have been developed for industrially used fungal species and strains in order to increase their enzyme production[9,10]. However, most of these methods are available for only a limited number of model or industrial fungi. In addition, the full capacity of fungi as enzyme factories depends on a detailed molecular level understanding of their physiology including regulatory mechanisms that govern enzyme production[2]. Although considerable progress has been made to improve the industrial potential of fungi, our knowledge remains limited and a number of questions have yet to be addressed. The availability of an increasing number of fungal genome sequences and omics data can lead to broader opportunities of genetic manipulations and research of potentially relevant industrial fungal species.

In the following sections, the approaches used for strain engineering of filamentous fungi in terms of production of enzymes and enzyme cocktails or plant biomass degradation will be discussed, using examples of the use of these strains to highlight their relevance and contribution to biotechnology. The availability of fungal (post-)genomics together

with modern genome editing technology has provided new opportunities for strain engineering that will be especially emphasized. The examples of strain engineering in fungi for enzyme production are numerous and it is impossible to provide a full overview of this topic. This review will therefore focus on the main approaches used for strain engineering, each accompanied by some examples of the specific enzymes or regulators controlling enzyme production. It should be noted that due to legislation issues, the implementation of some of these technologies is delayed in certain applications. As this is highly dependent on the country in which the processes are performed, we will not discuss restrictions of the use of the engineered strains caused by legislation in this review.

2. Classical strain engineering approaches

2.1 UV and chemical mutagenesis to improve enzyme activity

Mutagenesis approaches have been used to obtain strains with improved plant biomass degrading enzyme production. While this approach has been used for a variety of enzymatic activities, especially improvement of cellulase production has received extensive attention. To achieve this, better production strains have been obtained by random mutagenesis methods that are simple and easy to perform. Classical mutagenesis has been the most widely accepted method of strain improvement, and has also been used to create most of the fungal strains employed for commercial cellulase production, although a few have been generated through genetic modification[11].

One type of classical mutagenesis is the use of physical mutagens, such as ultraviolet (UV) radiation, which has proved to be an efficient approach for strain improvement. The UVC rays cause mutations as they pass through DNA and excite the atoms of the DNA molecule. The loss of electrons causes a change in the covalent bonds between DNA nucleotides and induces two adjacent pyrimidines, thymine and cytosine, to join and form a pyrimidine dimer (Figure 1A), most commonly resulting in point mutations. If this DNA damage is not repaired immediately, DNA polymerase replicates the mutation, resulting it to be present in both strands of the DNA. In most cases, UV mutations are very harmful, but may sometimes lead to better adaptation of an organism to its environment or in improved biocatalytic performance[12]. For example, when a fungus is exposed to mutagens at a sub-lethal concentration, the level of cellulase activity has been shown to increase[13,14].

A second type of classical mutagenesis is the use of chemical agents, such as intercalating molecules (e.g. ethidium bromide - EtBr), that can insert themselves between DNA strands and stretch the DNA duplex in such a way that DNA polymerase will insert an extra nucleotide opposite an intercalated molecule. Intercalating agents therefore typically cause frameshift mutations[15] (Figure 1B). In addition, alkylating agents, such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethylmethane sulphonate (EMS), have been used as mutagens in strain engineering. These chemicals add alkyl groups to nucleotides at various positions, which may lead to transition mutations where one pyrimidine or purine base is substituted by the other. The consequences of nucleotide mutations in protein coding regions of a gene depend on the substitutions of the nucleobases as well as the location of the mutations. This may lead to alteration in the enzyme amino acid sequence that may either increase, decrease or abolish the activity of mutant enzymes[15].

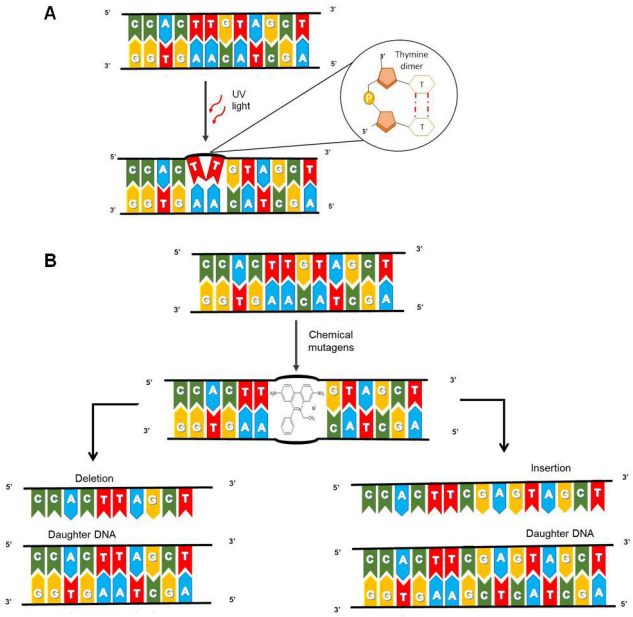

Figure 1. Classical mutagenesis. Changes that can be produced with these mutagens are depicted. **A**) UV light leads to the formation of thymine dimers, which can introduce frameshift or point mutations. **B**) For chemical mutagenesis, the chemical agent (e.g. ethidium bromide) is intercalated and introduces a spacing between base pairs, causing deletion or insertion of base pairs.

Use of UV radiation and chemicals, either separately or in combination, has been highly efficient in the generation of hypercellulolytic strains of filamentous fungi (Table 1). This is exemplified by the improvement of the cellulase titers of the *Trichoderma reesei* wild type strain QM6a (previously named *Trichoderma viride* QM6a)[16], resulting in strains with superior qualities in terms of cellulase activity levels, protein secretion and catabolite derepression, which have been used at the industrial level[17]. First, the mutant QM9414 with four-fold increase in cellulase production and two-fold increase in extracellular protein level compared to the wild type QM6a strain was generated by UV irradiation of conidia[18,19]. After this, UV mutagenesis and screening for catabolite derepression resulted in the isolation of strain M7[20]. A partially derepressed strain NG14 with increased production of extracellular protein and cellulase activity was obtained by chemical mutagenesis using N-nitroguanidine. Finally, the hypercellulolytic RUT-C30 strain was obtained after another round of UV mutagenesis together with screening for cellulase activity and catabolite derepression[21].

Other examples of increased cellulase production by classical mutagenesis were reported for another industrially used filamentous ascomycete, *Aspergillus*. A wild type *Aspergillus* strain was improved by two sequential treatments of Co^{60} γ-irradiation, UV irradiation and four sequential treatments with NTG[22]. This resulted in a mutant strain that produced 2-, 3.2- and 1.8-fold higher activity of carboxymethyl cellulase (CMCase), filter paper cellulase (FPase) and β-glucosidase, respectively, compared to the wild type[22]. Similarly, an *Aspergillus niger* mutant obtained by UV irradiation showed 3- and 2-fold increase in FPase and CMCase activity, respectively, compared to the parental strain[12]. Exposure of *Aspergillus oryzae* NRRL 3484 to sequential UV irradiation treatments followed by chemical treatments with NTG or EtBr, resulted in a mutant with a 4-fold higher FPase and CMCase activity than the wild type strain[23].

The general suitability of classical mutagenesis is supported by its use for the improvement of cellulase production in several other fungi that are less commonly or not used in industry. A UV mutant strain of *Penicillium echinulatum* had a high filter paper activity (FPA) compared to the wild type strain[24], while successive mutagenic treatments with EMS followed by UV irradiation generated a *Penicillium janthinellum* mutant that showed 3-fold FPase and 2-fold CMCase activity levels compared to the parent strain[25]. UV treatment followed by chemical mutagenesis using NTG also generated a mutant of *Fusarium oxysporum* with 80% higher cellulolytic activity than its parent strain[26]. In addition to the improvement of cellulase production, classical mutagenesis has also been used to improve the production of other industrially relevant enzymes in fungi. For example, an *Aspergillus tubingensis* mutant generated by UV mutagenesis demonstrated

improved xylanase activity, which was shown to be the result of a metabolic mutation[27]. In addition, UV irradiation has been successfully used to obtain *A. niger* mutants with increased pectinase production[28], whereas *A. niger* mutants showing improved lipase activity were generated with sequential exposure to UV radiation or nitrous acid[29]. Of the biotechnologically interesting fungal oxidative enzymes, increased laccase production has been achieved with EtBr treatment in the basidiomycete fungi *Cyathus bulleri*[30] and *Pleurotus citrinopileatus*[31].

These examples demonstrate the value of classical mutagenesis for improved enzyme production in filamentous fungi, and many of the mutants used at the industrial level have been obtained through this methodology. However, the use of classical mutagenesis also has disadvantages, in particular related to the non-targeted nature of this approach. The high dose of UV or chemical often used in industrial strain improvement strategies makes the generation of strains with single mutation highly unlikely. Improved production strains can acquire point mutations over several rounds of random mutagenesis leading e.g. to a reduced growth rate, sporulation defects or genomic instability. Some of these disadvantageous mutations will show up already in the screening of the progeny, and therefore strains containing them are likely discarded at this stage. However, other negative mutations may not show themselves until later stages of testing of the strains, such as reduced fermentation properties of the strain or reduced suitability for upscaling. Progeny with such negative characteristics would still be discarded, despite the amount of work already invested in them. In this context, directed genome manipulation can help to overcome the effect of deleterious point mutations[32].

Table 1. Classical strain engineering approaches. This table includes some examples of the improvements obtained in the production of enzymes in filamentous fungi that were engineered by classical methods.

Species	Improvements	Method*	References
Aspergillus niger	2-fold increase in FPase and CMCase activity	UV mutagenesis	[12]
Aspergillus oryzae	4-fold increase in FPase and CMCase activity	UV mutagenesis Chemical mutagenesis (NTG)	[23]
Aspergillus tamarii	Increased pectinase activity from 59 U/ml to 65 U/ml	UV mutagenesis Chemical mutagenesis (Hydrogen peroxide)	[33]
Aspergillus terreus	2- to 5-fold increase in CMCase, avicelase, FPase and β-glucosidase activity	UV mutagenesis Chemical mutagenesis (EMS)	[34]
Chaetomium cellulolyticum	1.6-fold increase in CMCase activity	γ-irradiation	[35]
Fusarium oxysporum	80% higher cellulolytic activity	UV mutagenesis Chemical mutagenesis (NTG)	[26]
Gliocladium virens	Higher β-1,4-glucosidase, CMCase and FP cellulase activity	UV mutagenesis Chemical mutagenesis (EMS)	[36]
Humicola insolens	Increase of 115% in CMCase, 303% in FPase and 196% in β-glucosidase activity	UV mutagenesis Chemical mutagenesis (MNNG)	[37]
Penicillium echinulatum	High FPase activity	UV mutagenesis	[24]
Penicillium janthinellum	3-fold increase in FPase and 2-fold increase in CMCase activity	UV mutagenesis Chemical mutagenesis (EMS)	[25]
Penicillium oxalicum	2.7-fold increase in CMCase activity	UV mutagenesis Chemical mutagenesis (EtBr)	[38]
Trichoderma reesei	2.7-fold increase in protein secretion, and 2.8-fold increase in FPase activity	UV mutagenesis Chemical mutagenesis (N-nitroguanidine)	[39]

*Only the methods used are mentioned, the methodology and the order in which the methods were used varies in each case and in many of them the strains were subjected to successive mutation steps. FPase = filter paper activity, CMCase = carboxymethyl cellulase activity, NTG = N-methyl-N'-nitro-N-nitrosoguanidine, EMS = ethylmethane sulphonate, MNNG = N-methyl-N-nitro-N-nitrosoguanidine, EtBr = ethidium bromide.

2.2 Genetically modified strains

After the discovery of DNA-mediated transformation procedures in the 1970s and 1980s, more targeted strategies were developed for the production of commercially valuable microbial strains. In addition, whole genome sequencing projects opened the possibility of genome mining, revealing a huge number of yet to be characterized genes encoding candidate plant biomass degrading enzymes[40–42]. Together with the development of novel molecular tools, such as homologous recombination (HR) and RNA interference (RNAi), genetic engineering of fungal strains became a commonly used approach for the development of strains with improved characteristics. Both forward and reverse genetics have been used to improve fungal production of plant biomass degrading enzyme production. Two early examples of forward genetics are the identification of the starch- and xylan-related transcriptional activators, AmyR from *A. oryzae*[43] and XlnR from *A. niger* [44], respectively. Examples of reverse genetics are also found for regulatory genes involved in the production of plant biomass degrading enzymes, such as *clr-1* and *clr-2* from *Neurospora crassa*[45], *rhaR* from *A. niger*[46] and *gaaR* from *Botrytis cinerea*[47].

One of the genetic engineering strategies to manipulate the enzymatic spectrum of fungal strains is the introduction of additional gene copies. In *T. reesei*, the introduction of β-glucosidase genes from other fungi, such as *Penicillium decumbens*[48], *Aspergillus aculeatus*[49] and *Chaetomium atrobrunneum*[50], was able to compensate for the low native β-glucosidase activity in this species. In *Penicillium oxalicum*, overexpression of β-glucosidase encoding genes improved the activity of this enzyme in culture filtrates 65-fold[51], while in *Humicola insolens* overexpression of a major cellulase gene (*avi2*) resulted in an 8-fold higher Avi2 activity[52]. Also other genes contribute to cellulose degradation efficiency. It was shown in the dung fungus *Podospora anserina*, which produces enzymes that act synergistically with those of *T. reesei*[53], that inactivation of cellobiose dehydrogenases reduced its ability to degrade cellulose[54].

Another application of genetic engineering for strain improvement is the manipulation of promoters that drive the expression of enzyme encoding genes and therefore affect enzyme production. Replacing the binding sites for the major carbon catabolite repressor protein CRE1, in the *cbh1* promoter by binding sites for the transcription activators ACE2 (a cellulolytic activator) and the HAP2/HAP3/HAP5 complex (a general expression enhancer regulator) in *T. reesei*, enhanced transcription of a test gene (green fluorescent protein) under cellulase inducing conditions 7-fold[55]. Similarly, introduction of additional copies of the enzyme encoding genes under control of string promoters can also improve enzyme production. Overexpression of cellobiohydrolases (*cbhI* and

cbhII) in *T. reesei* under control of the *T. reesei cbh1* promoter achieved a 1.3- to 4-fold overexpression[56], while overexpression of its *bgl1* gene under control of the *egl3* or *xyn3* promoter, increased β-glucosidase activities by 4.0- to 7.5-fold[57].

Combining the introduction of several genes can further enhance the effectivity of an enzyme mixture. Simultaneous expression of *bglI*, encoding a β-glucosidase from *A. niger* (AnBGL), and *eglIV*, encoding a lytic polysaccharide monooxygenase (LPMO) from *T. reesei* (TrLPMO), in *Penicillium verruculosum* under the control of the inducible *gla1* promoter resulted in more efficient hydrolysis of a lignocellulosic substrate than the control enzyme preparations[58]. Similarly, modification of the expression of a major regulator can also affect the enzyme mixture as a whole, such as the overexpression of *clrB* in *Penicillium oxalicum* using the *gpdA* promoter from *Aspergillus nidulans* that resulted in higher cellulase levels[59]. Similar approaches have been performed for other regulators, such as the major (hemi-)cellulolytic regulator of *A. niger* (XlnR), resulting in increased levels of xylanases and cellulases[60]. A combination of overexpression or activation of XlnR/Xyr1 and deletion of the major carbon catabolite repressor CreA/Cre1, resulted in even higher levels of (hemi-)cellulolytic enzymes in *A. niger* and *T. reesei*[61]. Similarly, overexpression of the amylolytic regulator AmyR, resulted in higher glucoamylase and α-amylase levels in *A. niger*[62].

One of the drawbacks of genetic engineering in filamentous fungi has been the low frequency of targeted integration of the introduced gene. This is due to the fact that in these fungi DNA integration is mainly directed by non-homologous end joining (NHEJ), resulting in a very low frequency of site-specific recombination[63]. To improve this percentage, strains that are defective in NHEJ have been constructed in several fungal species. The NHEJ process is mediated by the DNA-dependent protein kinase catalytic subunit, the Ku70-Ku80 heterodimer, and the DNA ligase IV-Xrcc4 complex. A high percentage of homologous recombination is achieved when either the *ku70* or *ku80* gene is disrupted or deleted[63]. For example, deletion of the Ku70 homologue in *A. nidulans* (*nkuA*Δ) improved homologous integration from 13% to 90%[64]. Similar results have been reported for other fungi, such as *A. oryzae*[65], *Aspergillus fumigatus*[66] and *Magnaporthe grisea*[67]. In *Penicillium decumbens*, deletion of *ku70* improved the targeting event to even 100%[68]. The availability of this methodology has also allowed the construction of gene knockout libraries of *Neurospora* and *Aspergillus*, leading to the identification of several new transcription factors involved in regulation of the production of cellulases and hemicellulases[69–71]. There is a potential risk in using this approach as Ku proteins are important to maintain telomere length in yeast and plants, and are necessary to ensure chromosome stability in mammals[72,73]. Phenotypic analysis of fungal

strain defective in NHEJ demonstrated that these strains showed higher susceptibility to various toxins and irradiation[74]. A recent study in *T. reesei* used transient silencing of NHEJ, to prevent those negative effects of *ku*-deletions[75]. Such a transient system may be needed when applying removal of NHEJ in industrial production strains.

3. Novel strain engineering approaches

The availability of genome sequences and novel methodologies have strongly expanded the toolkit for fungal strain engineering, but also enable a higher level of control of the strain modifications as well as the analysis of the resulting progenies. In particular, highly precise genome editing technologies have broadened the range of modifications that can be done at a targeted locus. However, this would not be anywhere near as efficient without the current (post-)genomic methodologies that allow efficient design of genome editing approaches as well as detailed analysis of the resulting strains. These methodologies have also provided a better understanding of improved strains that are generated using non-targeted strain engineering methodologies (mutagenesis, evolutionary adaptation), providing additional leads for targeted strain engineering approaches.

3.1 CRISPR/Cas9 technology

3.1.1 Introduction to CRISPR/Cas9

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas) system originates from bacterial and archaeal immune systems that contain *cas* genes and CRISPR array(s), which consist of short sequences that originated from foreign genetic material (also called spacers) interspaced with identical palindromic repeats[76]. The proteins encoded by the *cas* genes are responsible for acquisition of new foreign sequences into the CRISPR array(s) as well as for disruption of exogenous DNA through the activity of Cas proteins bearing endonuclease activity, such as the Cas9 protein[77].

In the CRISPR/Cas9 system, the Cas9 protein forms a complex with two RNA molecules: CRISPR RNA (crRNA)[78], encoded by the random spacers found in the CRISPR array(s), and trans-activating CRISPR RNA (tracrRNA)[79]. It has been shown, that these two RNAs form a dual-tracrRNA:crRNA, which can also be designed as a

single guide RNA (sgRNA) for genome editing purposes[76]. The Cas9-sgRNA complex is guided to a target DNA via homology of the crRNA to the protospacer region of the target sequence (Figure 2). The Cas9 nuclease subsequently binds the target DNA through interaction with the protospacer adjacent motif (PAM) immediately downstream of the protospacer sequence. If the crRNA part of the sgRNA sequence successfully pairs with the target DNA, the Cas9 nuclease will perform a double strand break (DSB) three nucleotides upstream of the PAM motif[80], which can be repaired either by the NHEJ or the homology-directed repair (HDR) pathway. The simple design and construction of a single guide RNA for precise genome editing has rapidly expanded the toolbox of molecular methods, such as Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALENs)[81].

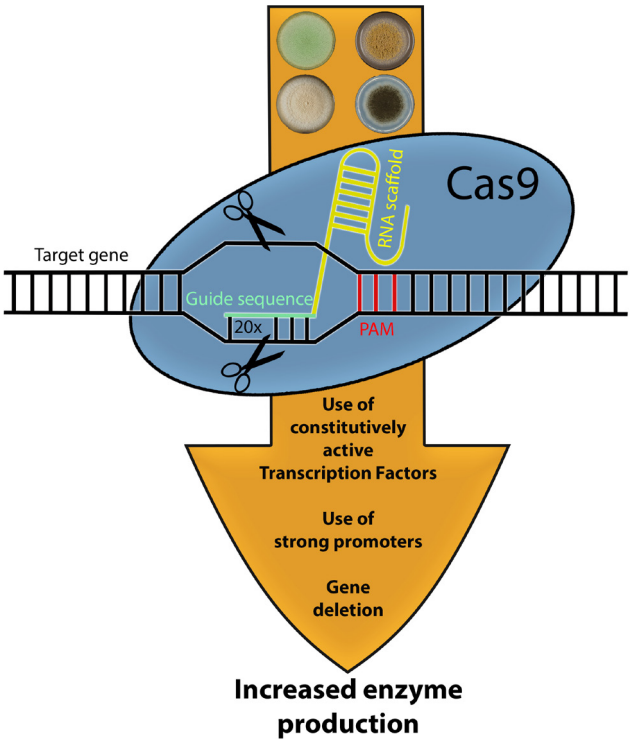

Figure 2. Utilization of the CRISPR/Cas9 system in filamentous fungi for increased lignocellulolytic enzyme production. The artificial sgRNA is composed of a guide sequence (in green) and a scaffold sequence (in yellow), which are the corresponding parts of the naturally occurring crRNA and tracrRNA, respectively. The sgRNA forms a complex with the Cas9 nuclease (in blue), which will be directed to a targeted locus of a gene of interest. In case there is sequence homology between the guide sequence and the target sequence upstream of a PAM sequence (in red), a double strand break (DSB) will be performed. The DSB can be repaired in a targeted manner using repair templates, which can result in precise gene editing.

The SpCas9 from *Streptococcus pyogenes* is the most widely used Cas9 nuclease due to the abundant presence of its target PAM sequence (5'-NGG-3')[76] throughout genomes of many types of organisms. This short, abundant PAM sequence provides many possibilities to edit the genes of interest, but it can also result in undesired mutations. The unintended mutations were mostly seen in organisms with large genomes, such as in human cell lines, where the CRISPR/Cas9 system has been reported to cause a wide variety of insertions, deletions and point mutations or even more complex genomic rearrangements[82–84]. However, there is no current evidence of complex CRISPR/Cas9-induced rearrangements in filamentous fungi. A comprehensive CRISPR/Cas9 off-target analysis would potentially decrease the probability of undesired mutations in these target organisms.

3.1.2 Implementation and improvement of CRISPR/Cas9 in filamentous fungi

CRISPR/Cas9 is a cost-efficient and simple platform to perform genetic manipulations with a single enzymatic activity guided by a pre-designed sgRNA molecule. Therefore, it has become a common genome editing method in a variety of organisms, such as yeast[85] and human cell lines[86,87], and has been reported to work in numerous filamentous fungi, including some basidiomycete species (Table 2). In filamentous fungi, this system has been used to either target genes involved in metabolite or enzyme production[88–99] or to establish a CRISPR/Cas9 genome editing system in a new species[96,100–105]. In some cases, CRISPR/Cas9 was tested in closely or even distantly related fungal species to demonstrate the versatility of the system[106].

In order to perform genetic manipulations with the CRISPR/Cas9 system, both the Cas9 nuclease and the sgRNA need to be present in the host organism. In fungi this can be achieved either through integration of *cas9* and sgRNA encoding constructs into the genome[101], through expression from a replicative plasmid encoding both the *cas9* gene and sgRNA[104], transient expression of the system from a non-replicating plasmid[96], or by using *in vitro* assembled ribonucleoproteins (RNPs)[105]. In some cases, the combination of *in vitro* and *in vivo* methods was applied with the utilization of *in vitro* synthesized sgRNA, while the *cas9* gene was either integrated into the genome of the host organism[103] or expressed from a replicative plasmid[93]. Additionally, genome editing in the mucoromycota species *Mucor circinelloides* was performed using *in vitro* synthesized sgRNA and purified Cas9 protein, but without *in vitro* RNP formation[107].

The first report about application of the CRISPR/Cas9 system in a filamentous fungus was for the induction of mutagenesis in the industrially relevant enzyme producer *T. reesei*[103]. The *cas9* gene of *S. pyogenes* was codon-optimized and fused with the SV40 nuclear localization signal (NLS), after which the resulting expression construct was randomly inserted into the genome of *T. reesei*. The *cas9* gene was expressed under the control of the constitutive promoter *Ppdc* or the inducible promoter *Pcbh1*, demonstrating that this system can work as an efficient and controllable genome editing tool in *T. reesei*[103]. Shortly after the initial successful application of the CRISPR/Cas9 system in *T. reesei*, Nødvig et al. published a self-replicating plasmid-based CRISPR/Cas9 transformation system, which was successfully applied in six *Aspergillus* species[104]. For this, four plasmids were constructed, each containing a different fungal selection marker, either an auxotrophic marker (*AFUM_pyrG* and *AN_argB*) or an antibiotic selection marker (hyg^R and ble^R)[104]. All these plasmids carried the *cas9* from *S. pyogenes*, which was codon optimized for *A. niger* and extended with the SV40 NLS[104], similar as in *T. reesei*[103]. Importantly, a key component of this system established in Aspergilli is the *ama1* gene from *A. nidulans*[108], which enables plasmids to autonomously replicate in many fungal species and therefore prevents the need for integration of *cas9* gene into the fungal genome. Moreover, this self-replicating plasmid based method allows the possibility of marker-free genome editing, enabling multiple editing steps using the same selection marker[104]. The sgRNA constructs were cloned into the CRISPR/Cas9 vectors in a single USER-cloning step[109], resulting in *in vivo* expression of the guides under the control of the strong constitutive *PgpdA* promoter and the *TtrpC* terminator[104]. Due to the lack of well-defined RNA polymerase III promoters in filamentous fungi, such as the U6 promoter[110], the sgRNA was released from a larger polymerase II transcript by the action of two ribozymes[104]. The plasmids of this study proved to be widely usable for genome editing in Aspergilli and even for the establishment of CRISPR/Cas9 transformation systems in phylogenetically distinct organisms[93,97,98,111–116].

The establishment of a CRISPR/Cas9 system in the industrially relevant ascomycete, *A. oryzae,* also provides possibilities for improved (heterologous) protein production[102]. While this system resulted in a low mutation rate (10-20%), it was demonstrated to work in two strains used for sake and soy sauce production, highlighting the opportunities for genome editing of industrially relevant strains[102].

It was recently shown that using NHEJ deficient strains of *A. nidulans, A. niger* and *A. oryzae*, successful gene targeting was achieved with single-stranded 90-mer oligonucleotides as repair templates of Cas9-induced DNA double-strand breaks[117]. This approach can be used to introduce precise modifications in the sequence of a

gene of interest. Moreover, it was reported that using the *A. fumigatus* U3 promoter to mediate expression of sgRNAs, multiple gene alterations could be performed at the same time, which facilitates complex genetic engineering in filamentous fungi[117].

Basidiomycete fungi are less commonly used in biotechnology applications, partly due to the lack of genetic transformation systems for most species and relatively poor behavior in submerged fermentations of many species. More recently, the use of solid state fermentation of basidiomycete fungi for biological pre-treatment has gained increased attention[118–120], which may soon result in increased attempts for strain engineering of these fungi. The availability of genome sequences has revealed the wealth of plant biomass degrading enzymes in basidiomycete fungi[42]. This has raised interest in the development of these fungi for applications, requiring efficient strain engineering methodologies. So far, genome editing in basidiomycetes has only been described for few species, including *Coprinopsis cinerea*[121], *Ganoderma lingzhi*, *Ganoderma lucidum*[122], *Ustilago maydis*[123,124] and *Ustilago trichophora*[125]. It is worth to mention that the *U. maydis* CRISPR/Cas9 system[123] involved the utilization of a plasmid carrying an autonomously replicating sequence (ARS)[126] responsible for self-replication, which is a similar approach to the one developed for ascomycete fungi[104]. In particular, wood-degrading white rot basidiomycetes are essential for efficient degradation of lignin[4] and the adaptation of a CRISPR/Cas9 transformation system in these organisms could facilitate the generation of hyperligninolytic strains for applications in which removal of lignin is needed.

Table 2. CRISPR/Cas9 systems in filamentous fungi. This table includes the establishment of a CRISPR/Cas9 transformation system in different species, the utilization of CRISPR/Cas9 mediated genome editing for enzyme/metabolite production or investigation of metabolic pathways.

Species	Purpose/Relevance	Origin of Cas9	Origin of sgRNA	References
Alternaria alternata	Establishment of CRISPR/Cas9 system	AMA1 plasmid		115
Aspergillus aculeatus	Establishment of CRISPR/Cas9 system	AMA1 plasmid		104
Aspergillus brasiliensis	Establishment of CRISPR/Cas9 system	AMA1 plasmid		104
Aspergillus carbonarius	Establishment of CRISPR/Cas9 system	AMA1 plasmid		104
	Comparison of gene targeting tools	AMA1 plasmid		116
Aspergillus fumigatus	Establishment of CRISPR/Cas9 system	Integrative		101
	Reconstitution of trypacidin production	Integrative		90
	Establishment of gene integration via microhomology	Integrative or AMA1 plasmid	Integrative, *in vitro* or AMA1 plasmid	127
	Application of RNP mediated transformation method	*In vitro* RNP[1]		128
	Off-target assessment	*In vitro* RNP[1]		129
	Investigation of azole resistance	*In vitro* RNP[1]		130
Aspergillus luchensis	Establishment of CRISPR/Cas9 system	AMA1 plasmid		104
Aspergillus nidulans	Establishment of CRISPR/Cas9 system	AMA1 plasmid		104
	Investigation of zaragozic acid A biosynthetic pathway	AMA1 plasmid		114
	Identification of a novel promoter for sgRNA[2] expression; Efficient gene targeting with oligonucleotides; Multiplex genome editing	AMA1 plasmid		117
Aspergillus niger	Establishment of CRISPR/Cas9 system	AMA1 plasmid		104
	Efficient gene targeting with oligonucleotides	AMA1 plasmid		117
	Galactaric acid production	AMA1 plasmid	*In vitro*	93
	Identification of enzymes involved in the D-glucuronic pathway	AMA1 plasmid	*In vitro*	112
			AMA1 plasmid	113
	Development of a gene integration system	AMA1 plasmid		131

Species	Purpose/Relevance	Origin of Cas9	Origin of sgRNA	References
Aspergillus niger	Identification of novel promoters for sgRNA[2] expression	Integrative		[132,133]
		AMA1 plasmid	AMA1 plasmid or integrative	[134]
	Study of a constitutive GaaR regulator involved in pectinase production	AMA1 plasmid	AMA1 plasmid or *in vitro*	[111]
Aspergillus novofumigatus	Establishment of CRISPR/Cas9 system; *In vivo* and *in vitro* reconstitution of the biosynthetic pathway of novofumigatonin	AMA1 plasmid	AMA1 plasmid	[97]
Aspergillus oryzae	Establishment of CRISPR/Cas9 system	Integrative		[102]
	Improvement of gene targeting efficiency in industrial strains; Activation of sclerotia formation	Integrative		[135]
	Demonstration of versatility of a system established in *Nodulisporium* sp.	Integrative	*In vitro*	[106]
	Efficient gene targeting with oligonucleotides	AMA1 plasmid		[117]
Beauveria bassiana	Establishment of CRISPR/Cas9 system	Integrative	*In vitro*	[136]
Blastomyces dermatitidis	Establishment of CRISPR/Cas9 system; Study of zinc metabolism for fitness	Integrative		[137]
Cordyceps militaris	Establishment of CRISPR/Cas9 system	Integrative	*In vitro*	[138]
Fusarium graminearum	Establishment of CRISPR/Cas9 system	Integrative		[139]
Fusarium oxysporum	Establishment of CRISPR/Cas9 system; Identification of the role of *bik1* gene in the synthesis of a red pigment, bikaverin	*In vitro* RNP[1]	*In vitro* RNP[1]	[99]
Myceliophthora heterothallica	Demonstration of versatility of a CRISPR/Cas9 system established in *Myceliophthora thermophila*	Integrative	Integrative	[94]

Species	Purpose/Relevance	Origin of Cas9	Origin of sgRNA	References
Myceliophthora thermophila	Establishment of CRISPR/Cas9 system;	Integrative		94
	Engineering of a hyper-cellulase producing strain Enhancement of amylolytic activity through overexpression of a major amylolytic enzyme or the *amyR* transcription factor encoding gene; Increased lignocellulase activites through deletion of *amyR*	Integrative		91
Neurospora crassa	Establishment of CRISPR/Cas9 system;	Transient expression from a non-replicative plasmid[3]		96
	Improved cellulase production through overexpression of *clr-2*			
Nodulisporium sp. (No. 65-12-7-1)	Establishment of CRISPR/Cas9 system	Integrative	Integrative or *in vitro*	106
Penicillium chrysogenum	Establishment of CRISPR/Cas9 system	AMA1 plasmid or *in vitro* RNP[1]	AMA1 plasmid, *in vitro* or *in vitro* RNP[1]	105
Pyricularia oryzae (*Magnaporthe oryzae*)	Establishment of CRISPR/Cas9 system	Transient expression from a non-replicative plasmid[3]		100
	Application of RNP[1] mediated transformation method	*In vitro* RNP[1]		140
Sclerotinia sclerotiorum	Establishment of CRISPR/Cas9 system	Integrative		141
Shiraia bambusicola	Establishment of CRISPR/Cas9 system	Integrative		88
	Identification of genes involved in the hypocrellin biosynthetic pathway	Integrative		88,89,92
Sporormiella minima	Demonstration of versatility of a system established in *Nodulisporium* sp.	Integrative	*In vitro*	106
Talaromyces atroroseus	Establishment of CRISPR/Cas9 system;	AMA1 plasmid		98
	Identification of a novel gene responsible for production of polyketide-nonribosomal peptide hybrid products			
Trichoderma reesei	Establishment of CRISPR/Cas9 system	Integrative	*In vitro*	103
	Identification of a novel transcription factor; Enhancement of xylanase activity and higher hydrolytic activity on pretreated biomass	Integrative	*In vitro*	95

Species	Purpose/Relevance	Origin of Cas9	Origin of sgRNA	References
Coprinopsis cinerea	Establishment of CRISPR/Cas9 system	Integrative	Integrative	[121]
Ganoderma lingzhi	Demonstration of versatility of a system established in *G. lucidum*	Integrative	*In vitro*	[122]
Ganoderma lucidum	Establishment of CRISPR/Cas9 system	Integrative	*In vitro*	[122]
Ustilago maydis	Establishment of CRISPR/Cas9 system	ARS plasmid		[123]
	Study of secreted proteins and development of CRISPR/Cas9 mediated multiplexing	ARS plasmid		[124]
Ustilago trichophora	Establishment of CRISPR/Cas9 system	Transient expression from a non-replicative plasmid[3]		[125]

[1]RNP = ribonucleoprotein

[2]sgRNA = single guide RNA

[3]The plasmids were presumably not integrated into the host genome

3.1.3 Applications of CRISPR/Cas9 for lignocellulolytic enzyme production

Due to the broad range of mutations that can be introduced using CRISPR/Cas9, soon after its establishment in filamentous fungi it was used for the improvement of the production of lignocellulolytic enzymes (Figure 2). Strain engineering to manipulate the regulatory system controlling lignocellulolytic enzyme production is an attractive way to not only understand the molecular mechanisms underlying production of these enzymes, but also to generate better enzyme production strains. Typically, these studies address species that are either industrial enzyme producers (e.g. *T. reesei*, *A. niger*, *Myceliophthora thermophila*) or well-established academic reference species (e.g. *N. crassa*). Some examples of this are given below.

The CRISPR/Cas9 system established in *T. reesei*[103] was recently used to study a negative regulator of xylanase activity, named SxlR[95]. The overexpression of this regulator resulted in reduced xylanase activity, but did not affect cellulase activity, while the deletion of *sxlR* gene resulted in a significant increase in expression of genes encoding GH11 endoxylanases. Similarly, in *A. niger* the auxotrophic *pyrG* marker containing plasmid[104] together with *in vitro* synthesized sgRNA were used to introduce a mutated version of the gene encoding the D-galacturonic acid regulator, GaaR, into the endogenous *gaaR* locus[111]. The modified *gaaR* gene carried a single point mutation causing a W361R amino acid change and resulting in a constitutively active form of GaaR, leading to inducer-independent production of pectinolytic enzymes[111].

The CRISPR/Cas9 system was also successfully implemented in the ascomycete *M. thermophila*[94], an industrially relevant thermophilic species used for high-temperature fermentations and production of thermostable lignocellulolytic enzymes[142]. The target genes for deletions included a carbon catabolite repressor *cre-1*, an endoplasmic reticulum stress regulator *res-1*, a β-glucosidase *gh1-1* and an alkaline protease *alp-1*[94]. The gene replacement frequency was 95%, which is much higher than the 20% frequency achieved in traditional transformations. In addition, this system was successfully applied for multiple simultaneous deletions, deleting up to four genes in one transformation event[94]. All these deletions contributed to the improved (hemi)cellulase activity, which reached 13.3-fold increased activity compared to the wild type strain[94]. This system was also used without modifications in *Myceliophthora heterothallica*, indicating the possibility of application in other related *Myceliophthora* species[94]. Recently, the same CRISPR/Cas9 system[94] was used for the improved production of amylolytic and (hemi) cellulolytic enzymes through rational design of *M. thermophila* strains[91].

The deletion of a major amylase gene Mycth_72393, was shown to result in 23.6% lower amylase activity on starch, while the overexpression of the same gene resulted in 35% increased activity, highlighting the essential role of this enzyme in starch degradation. Overexpression of the key amylolytic enzyme regulator encoding gene *amyR* increased the amylase activity by 30%, while the deletion of this gene resulted in 23.7% reduced amylase activity in liquid culture supernatant[91]. The deletion of *amyR* also resulted in 3-fold increase in CMCase and xylanase activity.

The filamentous fungus *N. crassa* was also genetically engineered with the CRISPR/Cas9 system in 2015[96]. The *cas9* gene was expressed under the control of the *trpC* promoter and terminator region from *A. nidulans* after integration into the genome of *N. crassa*. The Small Nucleolar RNA 52 (SNR52) promoter from the yeast *Saccharomyces cerevisiae*[143] was successfully used to overexpress the single-guide RNA targeting the *clr-2*. The *clr-2* encodes a core transcription factor involved in the regulation of cellulase expression[45]. By placing the *clr-2* gene under the control of a β-tubulin promoter, approximately two hundred-fold increase of *clr-2* mRNA expression was observed compared to the wild type strain, which consequently increased the expression of cellulase genes[96].

Overall, these results show that the CRISPR/Cas9 system has been efficiently applied in filamentous fungi to characterize transcription factors involved in the regulation of lignocellulose utilization and to increase the lignocellulolytic enzyme production. The establishment of this transformation system in a broad range of filamentous fungi can further increase the possibilities to generate more efficient enzyme producing strains suitable for industrial applications.

3.2 Adaptive evolution

In nature, fungi evolved to adapt optimally to their environment resulting in highly diverse physiologies for different species. Applying the possibility for adaptive evolution in strain engineering by repeated culturing on a selective medium is a relatively novel approach in fungal biotechnology. It was first demonstrated for the yeast *S. cerevisiae* by improvement of its ability to ferment D-xylose[144], and was then also applied to generate *S. cerevisiae* strains that efficiently co-fermented D-glucose, L-arabinose and D-xylose[145]. This method was also applied for other features of this species, such as improved glycerol production and sulfite tolerance[146], improved growth on glycerol[147] and acetic acid tolerance[148].

Less examples of the use of adaptive evolution have been reported for filamentous fungi, with the first being adaptive evolution of *Metarhizium anisopliae* towards strains showing robust growth at 37ºC[149]. A similar approach was later used to obtain *A. nidulans* strains with increased growth rate due to adaptation to growth on solid media[150]. More recently, adaptive evolution has been shown to enable higher production of plant biomass degrading enzymes in *Aspergillus* species.

A. niger grows poorly on pure cellulose, but successive growth of this species on agar plates with cellulose as the only carbon source resulted in significantly improved growth and sporulation[151]. Analysis of the best mutant demonstrated increased cellobiohydrolase and β-glucosidase activity, while transcriptome analysis revealed reduced expression of the ortholog of the cellulase repressor of *Podospora anserina*[152]. The role of this gene in repressing cellulase production was confirmed by deleting it in *A. niger*, resulting in increased levels of cellobiohydrolase and β-glucosidase activity[151].

Similarly, successive culturing of *A. oryzae* on agar plates with inulin resulted in significantly improved growth of the progeny[153]. Interestingly though, the best mutant did not only display increased inulinase activity, but also several other plant biomass degradation related activities. The molecular basis for this change is not yet clear, but may for instance be caused by an increased overall secretion capacity.

These examples indicate the high potential of adaptive evolution for strain engineering, especially when GMO approaches are not desired, such as in food-related applications.

3.3 Incorporation of omics technologies into fungal strain improvement

In the post-genomic era, the development of high-throughput analyses has proven them to be powerful tools to enhance our understanding on complex biological systems[154]. The current omics approaches include genome sequencing, global transcriptomic profiling, proteomics and metabolomics, which allow a deeper examination of all components, interactions and functional states of the biological molecules in the cell. These new methodologies also provide a novel approach to strain engineering, not only in the analysis of the progenies of both forward and reverse genetics, but also in more strategic options to approach strain engineering.

Many of the omics methodologies were first implemented in the yeast *S. cerevisiae*. While *S. cerevisiae* has no significant ability to degrade plant biomass, we include some

of the strain improvement studies of this species here, as they provide examples of the potential of omics methodologies that can be also applied to engineering of filamentous fungi. *S. cerevisiae* is a glucose fermenting species, which has been intensively used by the bioethanol industry. However, it is unable to utilize many compounds derived from the hydrolysis of lignocellulosic biomass, such as pentoses (D-xylose, L-arabinose) and the disaccharide cellobiose[155]. To broaden its applicability, a large amount of research has been focused on engineering *S. cerevisiae* to convert xylose to ethanol[156–158]. For example, two *S. cerevisiae* strains, TMB 3399 and 3400, were described that were both able to catabolize and ferment D-xylose to ethanol[159]. These recombinant strains were constructed by chromosomal integration of the genes encoding D-xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK). *S. cerevisiae* TMB 3400 showed a 5-fold increase in growth rate and lower xylitol production than *S. cerevisiae* TMB 3399 when both were cultivated on D-xylose under oxygen limitation and anaerobic conditions. Subsequently, mRNA expression levels were compared in these strains showing a higher expression of a hexose transporter encoding gene *hxt*, a xylulokinase encoding gene *xks*, and genes *sol3, gnd1* and *tkl1* encoding enzymes involved in the pentose phosphate pathway[159]. These early studies can be considered as first steps towards a more profound understanding of *S. cerevisiae* metabolic engineering and gene expression analyses, which strongly facilitate the strategies for strain improvement.

The lignocellulose pretreatment for the production of 2nd generation biofuels generates several inhibitory compounds that prevent fungi and their enzymes to efficiently hydrolyze the substrate. Comparative transcriptome analysis has been used to engineer *S. cerevisiae* strains with increased tolerance to inhibitors derived from lignocellulose[160]. More recently, a better fermentation performance was reported for a mutant strain of *S. cerevisiae* that is tolerant to acetic acid and furfural originating from lignocellulose by applying comparative proteomics and metabolomics analyses together with high-throughput phenotyping[161]. Changes were observed in the maintenance of energy and redox homeostasis as well as in the minimization of stress-induced cell damages.

As mentioned above, manipulation of regulators is an attractive approach of strain engineering to improve production of lignocellulolytic enzymes. The regulatory system driving this process is complex, including several transcription factors that respond to different inducers[162]. Omics analyses can provide a comprehensive understanding of signal compounds and key transcriptional regulators, which may improve the engineering of industrial strains for higher productivity of target enzymes. A first requirement for this is the availability of genome sequences of relevant species, such as members of the genus *Aspergillus*. In 2005, the genome sequence of *A. oryzae*[163], *A. nidulans*[164]

and *A. fumigatus*[163] were released. Some years later, the number of published and annotated fungal genomes increased exponentially[40,165–168]. As model organisms for basic research and major species for biotechnology, there is a considerable body of literature on Aspergilli and the recent advances in post genomic analyses has generated new knowledge for strain engineering[169–173]. Genomics and post-genomics studies have accelerated progress in plant biomass degradation related research in these fungi, facilitating e.g. the discovery of novel regulators[46,70,174,175] and enzymes[176–180]. Some of these studies demonstrated the high variation in enzyme sets produced by *Aspergillus* species during growth on plant biomass, despite relatively similar genome content with respect to these enzymes[40,181]. Currently, all species of the genus *Aspergillus* are being sequenced, with the first section recently published[182], providing an unprecedented view into the diversity of a fungal genus. The differences in the plant biomass degrading approaches of Aspergilli revealed in these studies are perfect starting points for strategic strain improvement strategies for specific applications.

Omics techniques and data have already been used in several studies for strain improvement. Overexpression of gene encoding D-galacturonic acid responsive regulator GaaR in *A. niger* increased the transcription of genes encoding pectinases, D-galacturonic acid transporters and enzymes of the D-galacturonic acid pathway even under non-inducing conditions[111]. Proteomic analysis of the *gaaR* overexpression strain showed high level of pectinases secretion when cultivated in fructose. The further deletion of the main carbon catabolite repressor gene *creA* also improved pectinase production. This modified *A. niger* strain with high pectinase production capacity showed high potential for industrial applications.

In another study, an *A. nidulans* hexokinase/glucokinase (*hxkA1*/*glkA4*) mutant was generated in order to prevent hexose consumption through glycolysis[183]. A triple mutant was obtained through sexual crosses by combining these mutations with a deletion in *creA* (*creAΔ4 hxkA1 glkA4*). Transcriptomic and metabolomic analyses were performed to examine changes in gene expression profiles and identify metabolic profiles related to sugar catabolism. The results showed that the deletion of *creA* combined with blocking glycolysis resulted in an increased expression of two genes from pentose catabolic pathway (PCP) and five genes from the pentose phosphate pathway (PPP). In addition, several glycolytic genes were downregulated in both double and triple mutants when the mutant strains were grown on starch and cellulose. This strongly suggests that blocking glycolysis caused an initial negative feedback of D-glucose release and activated alternative metabolic conversion of this sugar and indicates that metabolic engineering of fungi for biotechnology applications will need to take into account additional pathways

to obtain the desired result.

Re-annotation of the CAZy gene content of the *T. reesei* genome in combination with the gene expression analysis in the presence of different carbon sources has identified uncharacterized enzymes and new insights on enzymes needed for plant polysaccharide degradation[184]. Strain engineering of the industrial lineage of *T. reesei*, in order to include those enzymes in the commercial mixtures, is likely to increase the efficiency of the mixtures for plant biomass degradation. A gene co-expression network analysis was also performed[185] based on transcriptome data of the *T. reesei* RUT-C30[186] in order to identify new target genes involved in sugarcane bagasse degradation. The *xyr1* gene encoding major positive regulator of cellulases and hemicellulases was co-expressed with 50 upregulated cellulase, hemicellulase and oxidative enzyme encoding genes. When the *ace1* gene, encoding ACE1 repressor involved in regulation of cellulase gene expression, was replaced with the endoglucanase gene *egl1* in *T. reesei* RUT-C30, an increased expression of cellulolytic regulators was observed[187]. Compared to the RUT-C30 strain, the mutant showed 90% and 132.7% increase in total cellulase and endoglucanase activities. Moreover, cellulases produced by the engineered strain were more efficient for hydrolyzing pretreated corn stover and Jerusalem artichoke stalk than those of RUT-C30.

Omics analysis has been widely used in other filamentous fungi as well. The genome of another industrially used fungus, *M. thermophila*, revealed a wide enzymatic repertoire including hydrolytic, oxidative and auxiliary enzymes[188], which offered a starting point for further investigation of molecular mechanisms and strain improvement[94,189,190]. Similarly, the availability of a full genome of *N. crassa* has allowed the identification of two essential transcription factors, CLR-1 and CLR-2, which are required for the expression of cellulolytic genes[45], and the identification of the ortholog (XLR-1)[191] of the previously identified (hemi-)cellulolytic transcription factor XlnR from *A. niger*[44]. Lately, chromatin immunoprecipitation (ChiPseq) and RNA sequencing were performed in order to identify biding regions for CLR-1, CLR-2 and XLR-1[192]. The results showed that CLR-1 bound to the regulatory regions of 293 genes in Avicel cellulose cultures, while CLR-2 bound to promoter sites of 164 genes in sucrose cultures when cellulase activity was not detectable. During growth on xylan, XLR-1 bound to the promoters of 84 genes, including genes encoding six hemicellulases, three acetylxylan esterases, one β-glucosidase and two β-xylosidases. The identification and functional analysis of these transcriptional regulators related to plant biomass degradation contributes to unraveling the molecular mechanisms underlying this process in filamentous fungi. Manipulation of transcriptional regulators is a highly promising approach for industrial strain engineering

as it targets the system as a whole, rather than individual enzyme activities.

Advances in genome sequencing and innovative high-throughput technologies have also revealed new insights into the molecular basis of plant biomass degradation by basidiomycetes. While strain engineering of basidiomycetes is still in its infancy, a number of omics studies have opened up insights towards further development of these fungi for biotechnology. This includes the identification of the enzymatic sets employed by basidiomycete fungi for plant biomass degradation[193–198], as well as the identification of the small molecular mass inducers of the regulatory systems controlling this process[199]. Recently, a comparative analysis of basidiomycete transcriptome datasets was reported[200], which showed that a large set of conserved CAZymes encoding genes are expressed in plant biomass related substrates, suggesting that these enzymes are critical for degradation of any plant biomass, and should therefore always be present in commercial enzyme cocktails.

3.4 Epigenetics in fungi and its potential for strain engineering

Typically, three types of genetic phenomena are considered under the heading epigenetics: chromatin remodeling through histone modification, DNA methylation and RNA interference. All three phenomena have been demonstrated in fungi, although not always all three in the same fungal species[201]. While no specific use of epigenetics for strain engineering have been reported, indication of its potential have been obtained[202]. In *T. reesei*, the nucleosomes -1 and -2 downstream of the activating element of the *cbh2* promoter are loosened under inducing conditions, making the TATA box accessible[203]. Interestingly, deletion of the xylanase regulator encoding gene (*xyr1*) in this fungus significantly reduced the chromatin opening[204]. This is likely due to control of the expression of 15 putative chromatin remodeling genes by XYR1. Chromatin accessibility in *T. reesei* also seems to be affected by the global carbon catabolite repressor protein CRE1, as a deletion or a truncated version of this regulator resulted in a more open structure of the chromatin in the promoter regions of *cbh1* and *cbh2*[205,206]. The direct role of chromatin structure was confirmed by a deletion of a histone acetyltransferase in *T. reesei*, which not only resulted in decreased growth and morphological changes, but also in strong reduction of cellulolytic genes under inducing conditions[207]. A similar result was obtained for a deletion strain of a histone lysine methyltransferase in *Pyricularia* (formerly *Magnaporthe*) *oryzae*, which resulted in significant reduction of the expression of a cellulase gene[208]. An aspect related to that is the fact that certain regions of the genome provide higher expression levels than others[202]. Identification of these regions

would provide better locations for genetic engineering, possibly ensuring higher enzyme production.

Indications for a role of DNA methylation in the expression of genes encoding plant biomass degrading enzymes have also been reported using an inhibitor of DNA methyltransferase, 5-aza-2'-deoxycitidine[209]. In the presence of this inhibitor xylanase activity was increased during growth on wheat bran, while expression of cellobiohydrolase and xylanase encoding genes was increased during growth on glucose. It should however be noted that results from studies with inhibitors should be interpreted with caution due to side specificities they may have[210].

While not studied in detail, the presence of antisense reads in transcriptomic studies of *T. reesei*[211] and *A. niger*[212], suggests the possibility of RNA interference as a regulatory mechanism in these fungi. This is further strengthened by the observation that genes with mainly sense transcripts on wheat bran and antisense transcripts on glucose included several encoding plant biomass degrading enzymes[212].

3.5 Selection and use of monokaryotic strains

Some fungi possess a sexual reproductive cycle, which provides an opportunity for strain selection and engineering through recombination during meiosis[213]. Dikaryotic fungal species produce monokaryotic offspring with diverse genetic combinations and therefore successive cycles of basidiome production and crosses can result in strain improvement without the need for mutagenesis[214]. Several examples of this have been reported, such as for the white rot species, *Trametes versicolor*, where a monokaryon grew better than its parental dikaryon on glucose-soy agar and hardwood kraft pulp[215]. Differences in laccase production were found for mono- and dikaryotic strains of *Pycnoporus cinnabarinus*[216], while this was also the case for laccases and other lignocellulolytic activities of *Pleurotus ostreatus*[217,218]. This offers interesting possibilities for strain improvement in these fungi, although the random nature of the genetic recombination may require the screening of an extensive set of progenies to obtain the best producing strain.

4. Selection of screening conditions

Irrespective of the approach chosen for strain engineering, the selection of the best

resulting strain largely depends on the screening strategy chosen for the progenies. The ideal screening approach should provide a high probability of obtaining the desired alteration with a low chance of side-effects, such as changes that could later show to be disadvantageous. Typically, screening approaches can range from relatively simple (e.g. growth profiling) to more complicated and laborious (e.g. PCR-based analysis) methods. The design of the screen is likely the most important aspect of any strain engineering strategy, although technical limitations may be enforced by the engineering approach. In this section, some examples of screening approaches applied to filamentous fungal strains are presented.

If the desired improvement results in a growth phenotype, a direct selection of the progenies based on fungal growth on the screening media can be applied. This is for instance possible when the aim is to obtain strains with higher xylanase activity. Growth of progeny of the fungi on agar plates with partially soluble xylan as a carbon source, will result in clearing around the colony, with the largest clearing diameter indicating the highest xylanase activity[219].

However, in many cases growth on selective medium is combined with an indicator of enzyme activity. These can be highly specific substrates, such as 4-methylumbelliferyl glycosides, that can be used to screen for specific enzyme activities by detecting fluorescence of the released 4-methylumbelliferone[220], but also staining of the screening/ growth substrate is commonly used. After growth on agar plates supplemented with carboxymethylcellulose (CMC), the plates can be flooded with Gram's iodine, resulting in a bluish-black complex with cellulose, while a clear zone is visible where cellulose has been hydrolyzed, thus indicating cellulase activity[221]. Use of Congo-Red will also provide a similar clear zone on CMC as an indication of cellulase activity[222], while screening for increased starch hydrolysis can be done on starch agar plates using remazol brilliant blue[223], and for polygalacturonase activity on polygalacturonic acid agar plates using cetyltrimethylammonium bromide[224].

When improving enzyme production in fungi, enzyme activity assay based screening methods are advantageous as they provide detailed information about the modification that occurred in the progenies. To screen strains with an improved ability to degrade plant biomass, the measurement of the amount of reducing sugars using dinitrosalicylic acid (DNS)[225] has become a commonly used method, and is highly suitable for a high-throughput (robotic) setup. The DNS method measures the overall release of sugars from the reducing-ends of oligo- and polysaccharides and is therefore not specific to a certain linkage of activity, but does exclude the detection of activity of oxidative enzymes, such

as lytic polysaccharide monooxygenases (LPMOs). Some specificity can be obtained by not using crude plant biomass as a substrate, but pure polysaccharides (e.g. cellulose, xylan, polygalacturonic acid)[226–229]. However, this will still measure the combined activity of all (endo- and exo-acting) enzymes that depolymerize the used substrate. This method is therefore particularly useful for the selection of strains in which a number of enzyme activities or overall plant biomass degrading ability is improved. Variations on this method have also been described, some of which have a higher sensitivity than the original method. For example, the use of the formation of osazones from reducing sugars and *p*-hydroxybenzoic acid hydrazide was reported to be 5-fold more sensitive than a typical DNS assay and could therefore be efficiently used in 96-well plates and with low enzyme loading[230]. The use of micro-plate cultures and enzyme activity assays also allowed for the relatively simple analysis of both cellulase and xylanase activity[231].

An example of a different method to screen for progenies is the use of the *cbh1* promoter in front of a gene encoding a fluorescent protein (DsRed) in *T. reesei*, which allowed for screening for overproduction of cellulases by fluorescence[232]. Recently, a novel screening method using micro-fluidics was reported, in which single spores of *A. niger* sorted by fluorescence-activated cell sorting (FACS) germinated and grew in 10 nL droplets and were suitable for fluorescence-based enzymatic screening, as demonstrated for strains with improved α-amylase activity[233]. This method may facilitate high-throughput low volume screening that would improve current approaches.

5. Future perspective

Fungal strain engineering has a long history, but as indicated in the previous sections, the possible approaches to obtain strains with improved performance for the production of plant biomass degrading enzymes has broadened significantly in recent years. This is due to the recently established methods such as CRISPR/Cas9 genome editing and the potential of implementing epigenetics. These tools are still being further developed to higher efficiency and are accompanied by improved fungal genome sequences, exemplified by the gold-standard genome for *A. niger*[234] and the recently initiated genome sequencing project of the Joint Genome Institute of the Department of Energy of the USA (https://jgi.doe.gov/csp-2019-finishing-genomes/). This diversity of possibilities for strain engineering will facilitate a more strategic choice in the best approach for a certain ultimate aim also keeping in mind legislation/public acceptance with respect to GMO methodology.

An important challenge is to make strain engineering applicable to a wider range of fungi, which requires the development of efficient genetic transformation systems for them. In addition, effective submerged or solid-state fermentation protocols are essential for application of fungi in industrial processes. With those hurdles removed, the potential of strain engineering will go far beyond the currently used fungal species and strains, and will likely significantly contribute to the establishment of a bio-based economy. Similarly, development of robotic screening methods for a wider range of enzyme activities would strongly stimulate selection of progenies, as this is now still quite laborious for several enzyme activities.

While combinations of different omics data (proteomics, transcriptomics, metabolomics) have already resulted in some deeper insights into the molecular mechanisms of plant biomass conversion of filamentous fungi[198,235–238], a strong development in this area can be expected in the coming years. This will not only be due to better correlations of such datasets, but also incorporation of other methodologies, such as ChIP-seq[238,239] and DAP-seq[240], which will provide many new leads for strain engineering.

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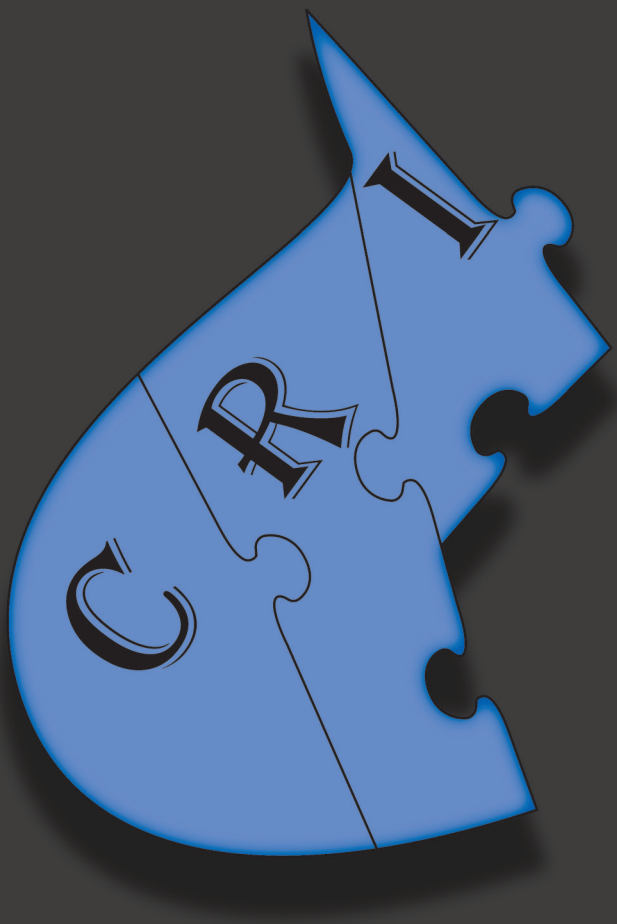
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Chapter 3

CRISPR/Cas9 facilitates rapid generation of constitutive forms of transcription factors in *Aspergillus niger* through specific on-site genomic mutations resulting in increased saccharification of plant biomass

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Abstract

The CRISPR/Cas9 system has been successfully applied for gene editing in filamentous fungi. Previous studies reported that single stranded oligonucleotides can be used as repair templates to induce point mutations in some filamentous fungi belonging to genus *Aspergillus*. In *Aspergillus niger*, extensive research has been performed on regulation of plant biomass degradation, addressing transcription factors such as XlnR or GaaR, involved in (hemi-)cellulose and pectin utilization, respectively. Single nucleotide mutations leading to constitutively active forms of XlnR and GaaR have been previously reported. However, the mutations were performed by the introduction of versions obtained through site-directed or UV-mutagenesis into the genome. Here we report a more time- and cost-efficient approach to obtaining constitutively active versions by application of the CRISPR/Cas9 system to generate the desired mutation on-site in the *A. niger* genome. This was also achieved using only 60-mer single stranded oligonucleotides, shorter than the previously reported 90-mer strands. In this study, we show that CRISPR/Cas9 can also be used to efficiently change functional properties of the proteins encoded by the target gene by on-site genomic mutations in *A. niger*. The obtained strains with constitutively active XlnR and GaaR versions resulted in increased production of plant biomass degrading enzymes and improved release of D-xylose and L-arabinose from wheat bran, and D-galacturonic acid from sugar beet pulp.

1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system originates from bacterial and archaeal immune systems. Cas proteins play a role in foreign sequence acquisitions, as well as disruption of exogenous DNA through endonuclease activity of some of these proteins, such as Cas9[1]. In the CRISPR/Cas9 system, Cas9 forms a complex with a CRISPR RNA (crRNA)[2], which originates from exogenous protospacer sequences, and a trans-activating CRISPR RNA (tracrRNA)[3]. Due to crRNA-protospacer homology, the Cas9-crRNA-tracrRNA complex will be directed to the target locus, where the Cas9 endonuclease interacts with the target DNA strand through a protospacer adjacent motif (PAM), unwinds the DNA strand, and performs a double-strand break three nucleotides upstream of the PAM[4]. This system was adapted for genetic engineering using designed synthetic single-guide RNAs instead of the original crRNA-tracrRNA complex[5] and it has been successfully applied in a variety of eukaryotic organisms[6–8], including efficiently plant biomass degrading filamentous fungi[9]. However, its application has mainly focused on the inactivation of genes through deletions, point mutations or on the insertion of genes at specific loci[9–12].

Plant biomass is the most abundant carbon source on earth and it consists mainly of plant cell wall polysaccharides (cellulose, hemicelluloses and pectin), and the aromatic polymer lignin. These polymers form a complex network, ensuring the strength and rigidity of plant cells[13]. The complex structure of plant biomass requires a broad set of hydrolytic and oxidative enzymes to degrade it. Filamentous fungi are efficient plant biomass degraders due to their ability to produce and secrete large amounts of Carbohydrate Active enZymes (CAZymes, www.cazy.org[14]). Fungal enzymes also have large variety of applications in many industrial fields such as food and feed, pulp and paper or textile and detergent industries[15].

The production of enzymes required for plant biomass degradation is regulated by transcription factors, which can act as transcriptional activators or repressors[16]. Many transcription factors have been described in ascomycetous fungal model organisms such as *Neurospora crassa*, and in organisms involved in industrial applications such as *Aspergillus niger, Aspergillus oryzae* and *Trichoderma reesei*[17].

The xylanolytic transcription factor XlnR from *A. niger* was the first described fungal regulator involved in (hemi-)cellulose utilization[18]. It was also shown that a single V756F point mutation in the C-terminal region of the *xlnR* gene results in a fully active transcription factor, even under repressing conditions[19]. Hasper et al. suggested that

mutations in the C-terminal region of XlnR disturb a putative inhibitory domain, which would normally turn this transcription factor into an inactive form[19]. A similar A871V point mutation in the C-terminal region of the *Penicillium oxalicum* XlnR ortholog also resulted in enhanced expression of lignocellulolytic enzymes[20].

The pectinolytic transcription factor GaaR was also reported to show constitutive activity caused by a single point mutation in *A. niger*[21]. The endogenous *gaaR* gene was deleted and replaced with a DNA construct carrying a W361R point mutation. Alazi et al.[21] proposed that this mutation disrupts the interaction between GaaR and its repressor, GaaX[22], under non-inducing conditions.

So far, attempts to generate constitutively active transcription factor mutants involved either site-directed mutagenesis of the target gene and its insertion in a specific genomic locus[19], the deletion of entire C-terminal regions of the target genes[23], or the insertion of a mutant allele in the deleted locus of the endogenous gene[21]. These are relatively labor-intensive approaches, which also may cause subtle additional changes at the site of integration or deletion that could further effect the phenotype. To demonstrate the versatility of CRISPR/Cas9-mediated genome editing, beyond the generation of loss-of-function deletions or point mutations and gene insertions, in this study we applied it for the generation of specific point mutations on-site in the native genomic copy of *xlnR* and *gaaR*, resulting in the previously reported constitutively active versions of the regulators. The exoproteomes of the mutant strains were evaluated by SDS-PAGE and enzyme activity analyses, and their ability to saccharify crude plant biomass substrates was assessed, to confirm the functionality of the mutated versions of the regulators.

2. Material and methods

2.1 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 ampicillin (Sigma Aldrich). Fungal strains used in this study were derived from the *A. niger* CBS 138852 strain. The generated mutants were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute under accession numbers indicated in Table 1.

Table 1. A. niger strains used in this study.

CBS number	Strain description	Genotype	Point mutations	Reference
CBS 138852	N593 Δ*kusA*	*cspA1*, *pyrG*$^{-}$, *kusA::amdS*		[24]
CBS 145907	XlnR V756F	*cspA1*, *pyrG*$^{-}$, *kusA::amdS*	G2330T	This study
CBS 145908	GaaR W361R	*cspA1*, *pyrG*$^{-}$, *kusA::amdS*	T1285C, C1293T	This study

Strains were grown at 30°C in Aspergillus Minimal Medium (MM) or Complete Medium (CM)[25] supplemented with 1% D-glucose and 1.22 g/L uridine (Sigma Aldrich).

For liquid cultures, 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, rinsed with MM, and approximately 2.5 g (wet weight) mycelium was transferred into 50 mL MM containing 0.45% D-fructose (corresponding to 25 mM) or 2% D-fructose, 1% wheat bran (WB) or 1% sugar beet pulp (SBP). Supernatant samples were taken after 24 h 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.

2.2 Construction of mutant strains

The ANEp8-Cas9-*pyrG* plasmid, which contains the autonomous fungal replicating element AMA1, *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[26]. The ANEp8-Cas9-*pyrG* plasmids (Figure S1) were constructed according to the protocol described by Song et al.[26]. The gRNA sequences were predicted using Geneious 11.1.4 software (https://www.geneious.com), and P1-P4 primers (Table S1) were used for the amplification of the gRNA expression constructs, which were cloned into the ANEp8-Cas9-*pyrG* plasmids and subsequently transformed into *E. coli*. Correct clones were identified by PCR amplification of the gRNA coding region by using the Fw-screen and Rev-screen primers (Table S1). All primers used in this study were ordered from Integrated DNA Technologies, Inc. (IDT, Leuven, Belgium).

Single-stranded DNA 60-mer, 90-mer or 200-mer oligonucleotides carrying specific point mutations (Table S2) (IDT, Leuven, Belgium) were designed to be used as repair templates to repair the double stranded DNA breaks caused by Cas9. Multiple templates were used for the introduction of GaaR W361R mutation, including templates

with extended length or multiple point mutations flanking the target site to facilitate a successful T → C transition in the nucleotide position 1285.

A. niger protoplasting and transformation were performed as described by Kowalczyk et al.[27], with minor modifications. One µg ANEp8-Cas9-*pyrG* plasmid, together with 5 µg of each corresponding repair template were used for each transformation. Putative mutant strains were purified by two consecutive single colony streaking, followed by cultivation on uridine-containing plates in order to remove the self-replicating AMA1 plasmid[28]. Candidates carrying the expected mutations were subsequently grown on medium containing 5-fluoro-orotic acid (5-FOA) in order to screen for colonies, which have lost the ANEp8-Cas9-*pyrG* plasmid. All *A. niger* mutants were confirmed by Sanger sequencing (Macrogen Europe, Amsterdam, the Netherlands) (Figure S2) using the sequencing primers listed in Table S1.

2.3 SDS-PAGE and enzyme activity assays

Culture filtrates of the control and mutant strains grown in media containing WB or SBP for 24 h were used to evaluate the produced extracellular CAZymes and their activities.

Twelve µL of the culture filtrates was added to 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), incubated at 85°C for 15 min, ice-cooled for 2 min and centrifuged at ~ 10,000 × *g* for 2 min. Finally, 10 µL were loaded onto 12% (w/v) acrylamide SDS-PAGE gels calibrated with PageRuler Plus prestained protein marker (Thermo Scientific), and silver stained[29] and documented using HP Scanjet G2410 scanner. All samples were evaluated in biological duplicates.

Enzyme activities were evaluated based on colorimetric para-nitrophenol (*p*NP) assays. Ten µL supernatant samples were mixed with 10 µL 0.1% 4-nitrophenyl β-D-xylopyranoside (for β-xylosidase activity), 0.1% 4-nitrophenyl β-D-galactopyranoside (for β-1,4-D-galactosidase activity) or 0.1% 4-nitrophenyl α-L-arabinofuranoside (for α-L-arabinofuranosidase activity) substrates, 50 µL 50 mM NaAc (pH 5) and 30 µL demineralized water in a final volume of 100 µL. β-xylosidase and β-1,4-D-galactosidase activities were measured after 1 h incubation at 30°C, while the α-L-arabinofuranosidase activity was measured after 30 min 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 both technical and biological triplicates. Differences in enzyme activities were determined using Student's two-tailed type II *t*-test. Significance was regarded as $p < 0.05$.

2.4 Saccharification tests

Saccharification reactions were performed in 96-well flat bottom microtiter plates. Each reaction contained 20 µL culture filtrate mixed with 50 mM sodium citrate (pH 5) containing 3% WB or 3% SBP in a final volume of 250 µL. The reactions were incubated for 6 h at 30°C and 400 rpm. Reactions were stopped by heat inactivation of enzymes for 15 min at 95°C. Plates were centrifuged for 20 min at 3220 × *g*, and the supernatants were 10-fold diluted in MilliQ water prior to analysis. The experiment was performed using biological and technical triplicates.

Monosaccharides were analyzed from peak areas in HPAEC-PAD (Dionex ICS-5000 + system; Thermo Scientific) equipped with CarboPac PA1 column (2×250 mm with 2×50 mm guard column; Thermo Scientific). The column was pre-equilibrated with 18 mM NaOH followed by a multi-step gradient: 0-20 min: 18 mM NaOH, 20-30 min: 0-40 mM NaOH and 0-400 mM sodium acetate, 30-35 min: 40-100 mM NaOH and 400 mM to 1 M sodium acetate, 35-40 min: 100 mM NaOH and 1 M to 0 M sodium acetate followed by re-equilibration of 18 mM NaOH for 10 min (20°C; flow rate: 0.30 mL/min). Between 5-250 µM D-glucose, D-xylose, L-arabinose and D-galacturonic acid (Sigma-Aldrich) were used as standards for quantification. Blank samples containing 3% WB or SBP, without the addition of culture filtrates were measured and the values were subtracted from each corresponding saccharification test result in order to exclude the amount of free sugar already present in the experimental condition. Differences in saccharification efficiency were determined using Student's two-tailed type II *t*-test. Significance was regarded as $p < 0.05$.

3. Results and discussion

3.1 CRISPR/Cas9 facilitates efficient on-site functional mutations

In order to achieve precise point mutations without unspecific genomic alterations, such as random insertions or deletions, we used *A. niger* Δ*kusA* as receptor strain for all our

transformations[24]. Due to the lack of non-homologous end joining (NHEJ) DNA repair pathway caused by the *kusA* deletion, Cas9 double strand breaks must be repaired with a repair template homologous to the target DNA region, facilitating the implementation of short templates carrying specific point mutations. The repaired DNA strand may still serve as a target region for further Cas9 cutting events, so due to the lack of NHEJ, it is important to introduce intended alterations of the protospacer or PAM sequence in order to avoid further double strand DNA breaks, leading to the death of the mutant colonies.

Nødvig et al. previously described that 90-mer single stranded oligonucleotides could be used for successful introduction of nonsense codons into the pigmentation gene *yA*, *alba* and *wA* of *A. nidulans, A. oryzae* and *A. niger*, respectively. It was also shown that the DNA repair did not show any preference for the targeted sense or anti-sense strand[30]. Based on this, we decided to use repair templates complementary to the anti-sense strand of the target DNA.

First, we performed a single GTC → TTC nucleotide mutation in the *xlnR* coding region, resulting in a valine-756-phenylalanine (V756F) mutation[19]. The gRNA sequence (P3-XlnR, P4-XlnR, Table S1) closest to the nucleotide of interest was predicted by Geneious. The 90-mer oligonucleotide repair template (XlnR repair template, Table S2) did not require any additional point mutations, since the target codon was also part of the PAM sequence, ensuring that the Cas9 endonuclease would not be able to re-bind and cut the repaired sequence anymore. After fungal transformation, three randomly chosen candidates were sequenced in their CRISPR/Cas9 target site (Figure 1A). All three candidates were shown to be correct and candidate 3 was randomly selected for further phenotype evaluation.

To obtain a constitutively active GaaR[21], a T → C transition in a TGG codon is required, resulting in a tryptophan-361-arginine (W361R) mutation. The gRNA sequence (P3-GaaR, P4-GaaR, Table S1) with the highest on-target activity was predicted by Geneious based on the experimentally determined predictive model proposed by Doench, et al.[31] Contrary to the *xlnR* point mutation design, an additional mutation was required in order to avoid re-cutting of the repaired target strand by the CRISPR/Cas9 system. Previous studies reported that the CRISPR/Cas9 system shows tolerance to mismatches in the protospacer sequence[32], which led us to alter the PAM sequence with a G → C silent mutation.

A 90-mer single stranded oligonucleotide was designed to introduce the intended point mutations, interspaced by 52 nucleotides (GaaR repair template 1, Table S2). After transformation, four randomly selected candidates were submitted for sequencing.

Sequencing results (Figure 1B) showed the intended alteration of the PAM sequence, although the W361R mutation did not take place. The same transformation was attempted with a longer 200-mer oligonucleotide repair template (GaaR repair template 2, Table S2). All sequenced colonies carried only the PAM sequence altering mutation (data not shown). Both attempts suggest that the repair templates were not entirely incorporated, excluding the W361R mutation, most likely due to the large distance between the two mutated nucleotides.

Taking into account these results, a re-designed gRNA encoding sequence (P3.2-GaaR, P4.2-GaaR, Table S1) closer to the target nucleotide was performed. Similarly to the previous approach, a new repair template was designed carrying two point mutations (2 PM), but this time the mutations were interspaced by only seven nucleotides. Since the new repair template carried both the intended W361R and the PAM sequence mutations closer to each other, we decided to reduce the length of the repair template to 60 oligonucleotides, which would theoretically induce homologous recombination (GaaR repair template 2 PM, Table S2).

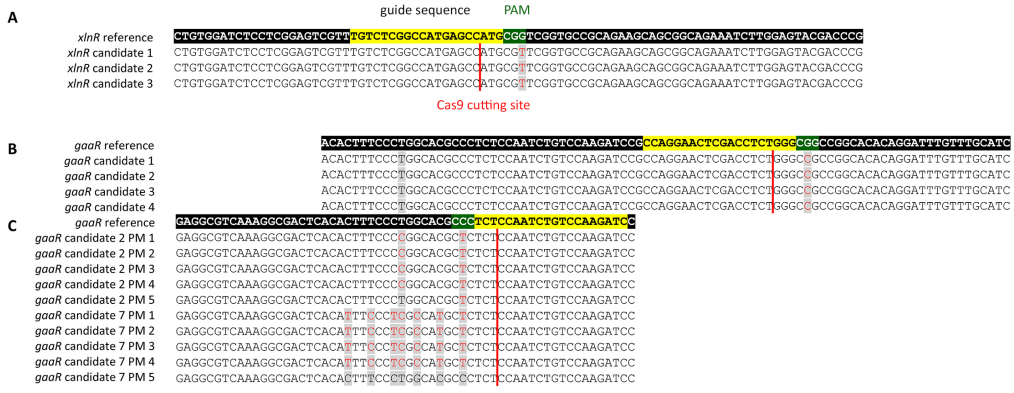

Figure 1. Sanger sequencing results of the XlnR V756F and GaaR W361R mutant candidates. **A**) Sequencing results of mutant candidates transformed with XlnR repair template. All three candidates showed the expected mutation. Sequences show the whole coverage of the used repair templates. Reference sequence is highlighted in black. The location of target nucleotides are highlighted in gray, while the introduced mutations are indicated in red. The PAM sequence is shown in green. The protospacer sequence is highlighted in yellow and the Cas9 cutting site is represented by a red bar. **B**) Sequencing results of mutant candidates transformed with GaaR repair template 1 (Table S2). All four sequenced candidates showed the introduction of the PAM sequence altering mutation. However, the W361R mutation did not occur. **C**) Sequencing results of mutant candidates transformed with GaaR repair template 2 point mutations (PM) or 7 PM (Table S2). Four candidates transformed with the 2 PM or 7 PM template showed the expected mutations. The 2 PM candidate 5 showed only the introduction of the PAM sequence altering mutation, while 7 PM candidate 5 showed a target sequence identical to the reference sequence. Results of (**B**) and (**C**) are aligned in order to emphasize the new guide RNA selection for the transformations using the 2 PM or 7 PM repair templates. Color codes as in (**A**).

In addition, another repair template was designed carrying five additional silent mutations (GaaR repair template 7 PM, Table S2) in order to hinder the homology of the sequence around the nucleotide of interest, to avoid the previously observed results where only the PAM sequence alteration occurred. Five transformant colonies were sequenced for each transformation, resulting in four correct mutants each (Figure 1C). Interestingly, the 2 PM repair template resulted in one colony carrying only the PAM sequence altering mutation, where most likely a recombination happened with ≤ 12 nucleotides serving as 5'-end flanking region, suggesting that even shorter repair templates could successfully restore the damaged DNA. This would be especially relevant when CRISPR/Cas9 genome editing is performed in an *A. niger* strain of a different lineage, whose genomic DNA sequence is likely not fully identical. The colony 2 PM 1 was selected for further phenotypic evaluation.

3.2 Constitutive versions of XlnR and GaaR result in elevated enzyme levels

Two crude plant biomass substrates were chosen for phenotypic characterization of XlnR V756F and GaaR W361R mutants. WB is rich in glucuronoarabinoxylan, suitable for characterization of a constitutive XlnR phenotype, whereas SBP has a high pectin content and was previously used for the characterization of *gaaR* deletion mutants[27,33].

SDS-PAGE and enzyme activity assays of 24 h culture filtrates after growth of the mutants and control strain on 1% WB and 1% SBP were assessed for phenotypic characterization. SDS-PAGE results of the XlnR V756F mutant grown in 2% D-fructose, a carbon source showing low carbon catabolite repression (CCR) mediated gene repression[34], showed the presence of mainly putative endoxylanases (predicted MW in the range of 13-33 kDa) and β-xylosidases (predicted MW is 122 kDa)[13] (Figure 2A), which were not present in the control strain, demonstrating the inducer-independent constitutive action of XlnR. The xylanolytic enzymes were more abundant when the mutant was grown in medium containing 0.45% D-fructose (Figure 2B), most likely due to the reduced CCR effect mediated by CreA compared to the 2% D-fructose culture[35,36]. Cultivation of the XlnR V756F mutant in 1% WB medium also resulted in an increase of the major putative xylanolytic enzymes compared to the control strain (Figure 2C), suggesting improved saccharification abilities in this mutant.

The cultivation of the GaaR W361R mutant in liquid medium containing 2% D-fructose as a sole carbon source did not result in an increased production of pectinolytic

enzymes as the SDS-PAGE pattern was identical to that of the control strain (data not shown). However, the samples from 1% SBP cultures showed elevated levels of CAZymes, especially in the 35-66 kDa range (Figure 2D), where most *A. niger* endo- and exopolygalacturonases, and pectin lyases are found[13]. The genes encoding these enzymes have been shown to be controlled by GaaR[37].

The SDS-PAGE profiles were confirmed by enzyme activity assays. β-xylosidase activity (BXL) was 53% increased in the XlnR V756F supernatant from 1% WB cultures compared to its control strain (Figure 3A). Moreover, BXL activity in the culture filtrate of this mutant showed very similar values when cultivated in 1% SBP, while the control strain did not show any BXL activity under this condition, due to the lack of activation of XlnR (Figure 3B). This result also proves that the XlnR V756F mutant can express its target genes in non-inducing conditions. Interestingly, the supernatant from GaaR W361R mutant showed a 31% increase in β-1,4-D-galactosidase activity (LAC) in WB medium. However, LAC activity was 63% reduced in samples of the SBP cultures, compared to the control. Since the regulation of β-galactosidase genes has been shown to be controlled by a broad range of transcription factors, including GaaR[37], the increase of LAC activity in WB medium and decrease in SBP medium may not be related to a direct constitutive GaaR effect, but rather to an altered interaction between the transcription factors controlling the production of this activity. Finally, the XlnR V756F mutant showed 15% and 10% reduced α-L-arabinofuranosidase activity (ABF) in the WB and SBP samples, respectively. In contrast, the GaaR W361R mutant showed a 22% and 6% increase in ABF activity in WB and SBP cultures, respectively, suggesting that the constitutive GaaR rather has an (minor) activating role in the expression of *abf* genes. Overall, the low fold change values suggest that neither XlnR V756F, nor GaaR W361R play an essential role in the activation of these genes, most likely because they are mainly controlled by the arabinanolytic regulator AraR[37].

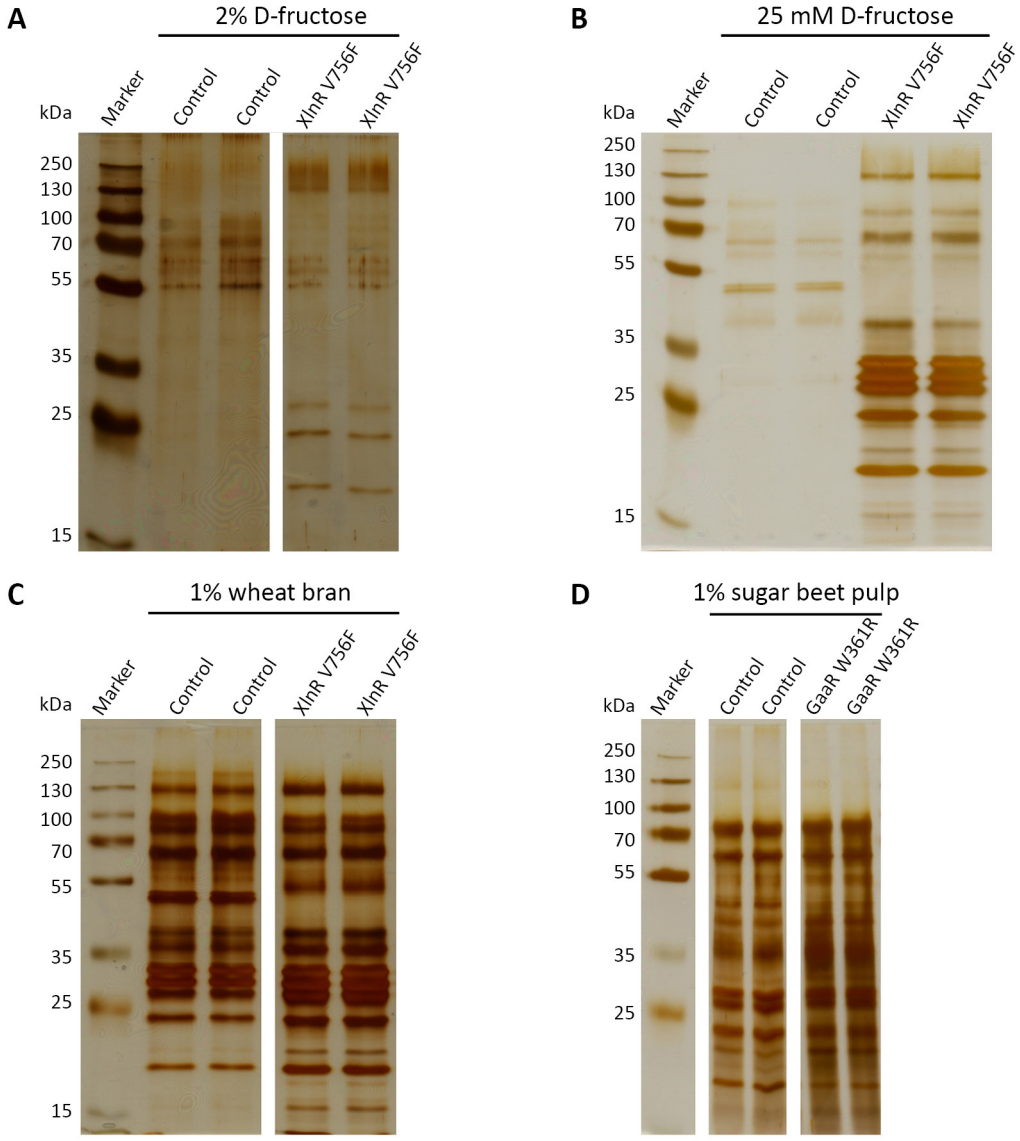

Figure 2. Enzyme production analysis of XlnR V756F and GaaR W361R mutant strains. SDS-PAGE analysis of the supernatants of *A. niger* CBS 138852 (control strain), XlnR V756F and GaaR W361R mutants after 24 h incubation in different culture conditions as indicated from **A-D**.

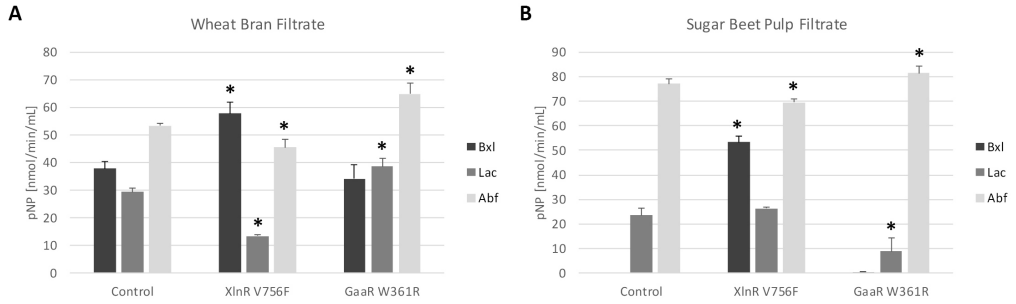

Figure 3. Enzyme activity of the supernatants from XlnR V756F and GaaR W361R mutant strains and from *A. niger* CBS 138852. The 24 h culture filtrates originated from 1% wheat bran (WB) (**A**) or 1% sugar beet pulp (SBP) (**B**). Different enzyme activities are indicated by gray scale color codes. The values represent the mean and standard deviation of the amount of released *p*NP measured at 405 nm wavelength. Experiments were carried out using biological and technical triplicates. Bxl = β-xylosidase, Lac = β-1,4-D-galactosidase, Abf = α-L-arabinofuranosidase. Statistical significance is represented by (*) ($p < 0.05$).

3.3 The enzyme mixtures from the constitutive regulator strains resulted in improved saccharification of wheat bran and sugar beet pulp

Saccharification tests were performed using the 24 h culture filtrates of XlnR V756F, GaaR W361R and the control strain cultured on 1% WB or 1% SBP (Figure 4) (subsequently referred to as WB culture filtrate and SBP culture filtrate, respectively). Both crude substrates were used in order to test the phenotype of each mutant strain under inducing and non-inducing conditions.

The release of D-xylose, L-arabinose, D-galacturonic acid and D-glucose from 3% WB and 3% SBP by the WB and SBP culture filtrates was measured. D-xylose release from xylan is regulated by XlnR, while the release of D-xylose from pectin has been suggested to be co-regulated by XlnR and GaaR[27,37]. The release of L-arabinose is mainly controlled by the arabinanolytic transcription factor, AraR[38], but XlnR and GaaR have also been reported to co-regulate some arabinanolytic genes[27,37]. The release of D-galacturonic acid from pectin is regulated by GaaR[33]. Finally, D-glucose can either be released from cellulose or starch. SBP is rich in cellulose, while WB contains both cellulose and starch[39]. Since neither XlnR nor GaaR was shown to play a role in starch utilization, D-glucose release would be most likely related only to cellulose utilization, in which XlnR is involved, as first suggested by van Peij et al[40].

Saccharification of WB (Figure 4) showed increased release of D-xylose (Figure 4A) and L-arabinose (Figure 4B) by the XlnR V756F mutant for both WB and SBP culture filtrates. When the SBP culture filtrates were used, D-xylose release by XlnR V756F was especially significant compared to the control strain, which did not release D-xylose due to the lack of XlnR induction (Figure 4A). The GaaR W361R mutant showed a similar amount of released D-xylose (Figure 4A) compared to the control for both culture filtrates, while L-arabinose release (Figure 4B) was similar for the WB culture filtrate, but reduced for the SBP culture filtrate. This could be associated with a competing effect between GaaR and XlnR[27], also supported by the fact that the constitutive XlnR resulted in a significant increase of L-arabinose release in this condition.

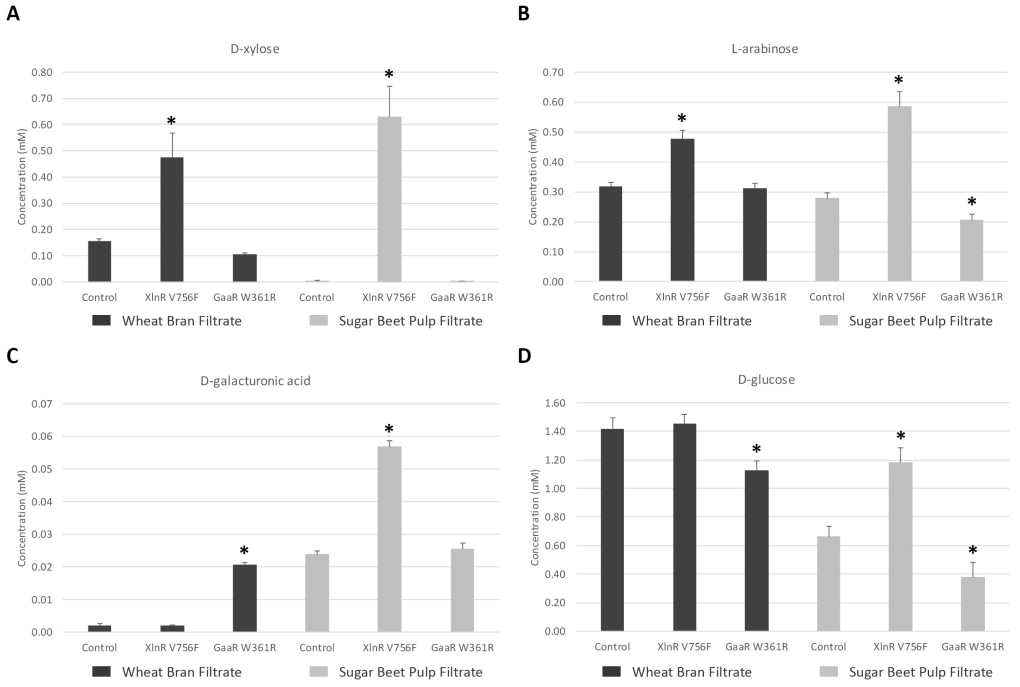

Figure 4. Monosaccharides released from 3% wheat bran (WB) by culture filtrates from control strain *A. niger* CBS 138852, and XlnR V756F and GaaR W361R mutant strains. The amount of D-xylose (**A**), L-arabinose (**B**), D-galacturonic acid (**C**) and D-glucose (**D**) released after 6 h incubations with WB or sugar beet pulp (SBP) culture filtrates are indicated by black and gray bars, respectively. Values represent the mean and standard deviation of sugar concentration indicated in millimolar (mM). Experiments were carried out using biological and technical triplicates. Statistical significance is represented by (*) ($p < 0.05$).

D-galacturonic acid is present in very low amounts in WB, most likely found only in thin layers of pectin located under the outer and the epidermal cuticles[39,41]. Our results show that SBP culture filtrate from XlnR V756F generated the highest D-galacturonic acid release (Figure 4C), probably due to the more abundant presence of β-xylosidases catalyzing the removal of D-xylose, which can decorate pectin. This could facilitate the degradation of the galacturonan backbone by the pectinolytic enzymes also present in the SBP culture filtrates. Regarding D-glucose release (Figure 4D), the XlnR V756F mutant showed a similar value compared to the control strain when WB culture filtrate was used. However, for the SBP culture filtrate, the XlnR V756F mutant showed significantly higher D-glucose release, which is most likely related to the improved xylanolytic activities, making cellulose more accessible for degradation in WB. Both GaaR W361R culture filtrates showed reduced D-glucose release from WB, possibly due to an antagonistic effect between GaaR and XlnR[27], resulting in decreased xylanolytic activity on hemicellulose in the constitutive GaaR mutant, thus reducing cellulose degradation.

In case of SBP saccharification (Figure 5), all strains released similar levels of D-xylose when WB culture filtrates were used (Figure 5A). In contrast, the XlnR V756F SBP culture filtrate showed a significant improvement in D-xylose release compared to the control. Regarding L-arabinose release (Figure 5B), SBP culture filtrates showed higher saccharification efficiency compared to that of WB culture filtrates. However, the mutants did not show improved L-arabinose release compared to the control strain, which indicates that other transcription factors, such as AraR, have a more predominant role in releasing L-arabinose from pectin. These results also suggest that the XlnR V756F and/or GaaR W361R mutants have a negative effect over AraR-mediated L-arabinose release from this substrate. In contrast, D-galacturonic acid release was significantly improved by both GaaR W361R culture filtrates (Figure 5C), especially in the case of WB culture filtrates, where the control strain released only a minimal amount of D-galacturonic acid due to the lack of GaaR induction. Finally, D-glucose release was similar for the WB culture filtrate samples, while both mutants showed a slight decrease compared to the control when SBP culture filtrates were used (Figure 5D).This suggests that neither XlnR nor GaaR have a major influence on cellulose degradation under these conditions.

Overall, the release of D-xylose and L-arabinose from WB was improved by the XlnR V756F mutant. D-xylose release from both WB and SBP using the non-inducing condition for XlnR (SBP culture filtrate) highlights the constitutive activity of the XlnR V756F mutant. D-galacturonic acid release from SBP was improved by the GaaR

W361R mutant, highlighting the constitutive activity in case of the non-inducing WB culture filtrates. The results of D-glucose release from SBP suggests that the cellulolytic activities of each sample are comparable, which justifies the results observed in case of WB saccharification, in which xylan degradation is the bottleneck of cellulose utilization.

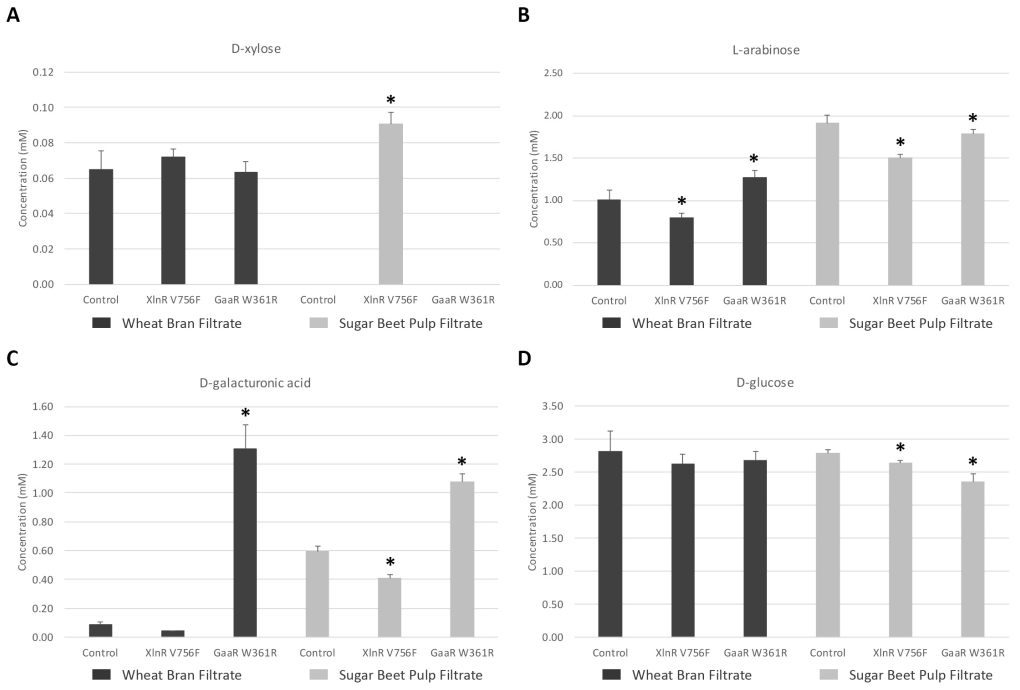

Figure 5. Monosaccharides released from 3% sugar beet pulp (SBP) by culture filtrates from control strain *A. niger* CBS 138852, and XlnR V756F and GaaR W361R mutant strains. The amount of D-xylose (**A**), L-arabinose (**B**), D-galacturonic acid (**C**) and D-glucose (**D**) released after 6 h incubations with wheat bran (WB) or SBP culture filtrates are indicated by black and gray bars, respectively. Values represent the mean and standard deviation of sugar concentration indicated in millimolar (mM). Experiments were carried out using biological and technical triplicates. Statistical significance is represented by (*) ($p < 0.05$).

4. Conclusions

In this work, we demonstrate how CRISPR/Cas9 genome editing can be used to efficiently modify the functionality of transcriptional regulators in *A. niger* by generating on-site mutation in the native copy of the corresponding genes in the genome. This also indicates that similar strategies could be used to change enzyme properties by mutating enzyme encoding genes, as well as many other functional mutations, further

expanding the versatility of this genome editing approach. We used single stranded 60-mer or 90-mer oligonucleotide-mediated gene editing to generate constitutively active XlnR and GaaR transcription factors, but our data suggests that even shorter fragments could be used as templates to repair the Cas9-induced DNA strand cuts. The XlnR V756F mutant secreted a higher amount of CAZymes involved in the release of D-xylose and L-arabinose from WB, confirming the functional mutation. Moreover, D-glucose release was also improved, likely facilitated by degradation of xylan, making cellulose more accessible for degradation in non-inducing conditions. Finally, the GaaR W361R mutant showed enhanced release of D-galacturonic acid from SBP. Overall, the use of CRISPR/Cas9 to generate such overproduction strains significantly reduced time and efforts compared to traditional approaches.

CRediT autorship contribution statement

Roland S. Kun: Investigation, Formal analysis, Writing - original draft. Jiali Meng: Investigation, Formal analysis. Sonia Salazar-Cerezo: Investigation, Formal analysis. Miia R. Mäkelä: Supervision, Writing - review & editing. Ronald P. de Vries: Conceptualization, Funding acquisition, Supervision, Writing - review & editing. Sandra Garrigues: Formal analysis, Supervision, Writing - review & editing.

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Supporting information

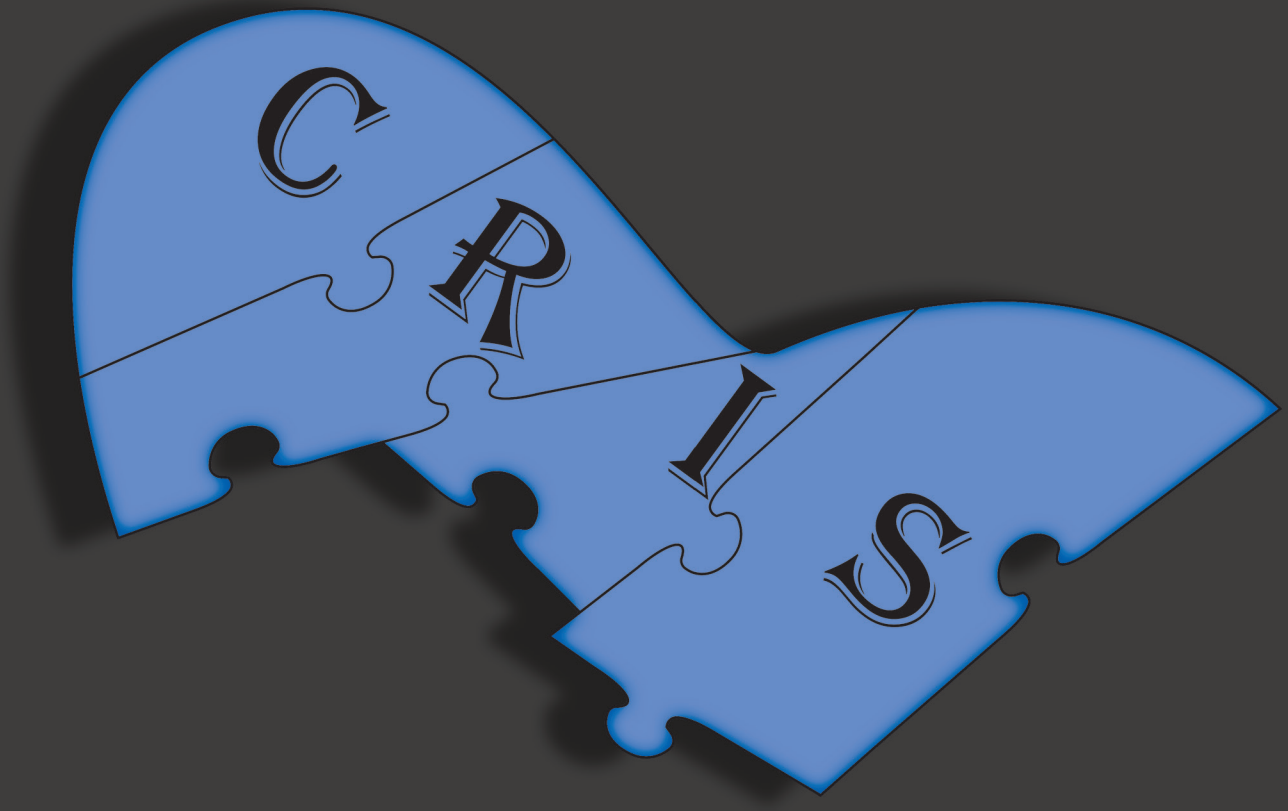
All supporting information are available at: https://doi.org/10.1016/j.enzmictec.2020.109508 or can be obtained upon request from the author.

Figure S1. Schematic representation of the ANEp8-Cas9-pyrG plasmid used to express the *cas9* gene. The *Swa*I restriction site required for gRNA integration is indicated on the plasmid map. Abbreviations: *bla*, beta-lactamase gene conferring ampicillin resistance; P*pkiA*, promoter of the pyruvate kinase gene; T*glaA*, terminator of the glucoamylase gene; AMA1, origin of autonomous replication; *pyrG*, orotidine 5'-phosphate decarboxylase gene.

Figure S2. Sanger sequencing results of screened mutants. Mutated nucleotides are highlighted in yellow. Peaks were visualized using SeqMan Pro software version 12.1.0 (141) (https://www.dnastar.com).

Table S1. Primers used in this study.

Table S2. Repair templates used for transformations.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Chapter 4

The chimeric GaaR-XlnR transcription factor induces pectinolytic activities in the presence of D-xylose in *Aspergillus niger*

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Roland S. Kun, Sandra Garrigues, Marcos Di Falco, Adrian Tsang and Ronald P. de Vries

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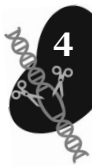
Abstract

Aspergillus niger is a filamentous fungus well known for its ability to produce a wide variety of pectinolytic enzymes, which have many applications in the industry. The transcriptional activator GaaR is induced by 2-keto-3-deoxy-L-galactonate, a compound derived from D-galacturonic acid, and plays a major role in the regulation of pectinolytic genes. The requirement for inducer molecules can be a limiting factor for the production of enzymes. Therefore, the generation of chimeric transcription factors able to activate the expression of pectinolytic genes by using underutilized agricultural residues would be highly valuable for industrial applications. In this study, we used the CRISPR/Cas9 system to generate three chimeric GaaR-XlnR transcription factors expressed by the *xlnR* promoter by swapping the N-terminal region of the xylanolytic regulator XlnR to that of the GaaR in *A. niger*. As a test case, we constructed a P*pgaX-hph* reporter strain to evaluate the alteration of transcription factor specificity in the chimeric mutants. Our results showed that the chimeric GaaR-XlnR transcription factor was induced in the presence of D-xylose. Additionally, we generated a constitutively active GaaR-XlnR V756F version of the most efficient chimeric transcription factor to better assess its activity. Proteomics analysis confirmed the production of several pectinolytic enzymes by Δ*gaaR* mutants carrying the chimeric transcription factor. This correlates with the improved release of D-galacturonic acid from pectin by the GaaR-XlnR V756F mutant, as well as by the increased L-arabinose release from the pectin side-chains by both chimeric mutants under inducing condition, which is required for efficient degradation of pectin.

1. Introduction

Filamentous fungi are efficient degraders of plant biomass, ensuring an essential role in the global carbon cycle. This is associated with their ability to produce and secrete large amounts of extracellular Carbohydrate Active enZymes (CAZymes, www.cazy.org)[1], which have a variety of applications in different industrial fields, including food and feed, pulp and paper or textile industries[2]. Fungal Zn_2Cys_6 transcriptional activators play a key role in the regulation of enzyme production by activating the expression of genes encoding for enzymes required for the degradation of the substrates found in the environment. However, these transcription factors need to be activated to express their target genes[3]. Thus, the availability of inducing compounds may be a limiting factor in industrial production processes.



Pectinases have a broad application in food industry, and are mainly used for juice clarification or in the production of jams, wine, coffee and tea[4]. *Aspergillus niger* has a long history of safe application and is often used in industry for the production of valuable metabolites and enzymes[5]. In particular, this fungus is well known to possess a large array of genes encoding pectinases and accessory enzymes[6]. A key transcription factor in the regulation of pectinase production is GaaR[7], which is activated by its physiological inducer 2-keto-3-deoxy-L-galactonate, an intermediate compound in the pathway of D-galacturonic acid catabolism[8] and has been shown to play a major role in the regulation of pectin degradation[9].

The alteration of transcription factor specificity through the generation of chimeric transcription factors has been reported more than thirty years ago in yeasts[10–12]. More recently, artificial transcription factors have been constructed in filamentous fungi to enhance the production of cellulases[13–18] or amylases[19]. However, the application of this technique to facilitate or enhance pectinase production has not yet been reported.

The xylanolytic transcription factor XlnR was the first (hemi-)cellulolytic transcription factor described in *A. niger*[20] and it is the most studied transcriptional activator involved in the regulation of plant biomass degradation. It is involved in the colonization of plant biomass and in the degradation of its components such as xylan and cellulose[20–22]. Moreover, D-xylose has been shown to activate XlnR[20], making this transcription factor a suitable candidate for the generation of D-xylose-inducible chimeric transcription factors. The generation of a GaaR specific chimeric transcription factor that could be induced when cultivated on (hemi-)cellulose-rich agricultural waste materials would be a suitable approach for the industry, due to the high abundance of these substrates in nature.

In this study, we used CRIPR/Cas9 genome editing[23,24] to generate a chimeric GaaR-XlnR transcription factor through on-site modification of the endogenous *xlnR*. As a test case, we generated a hygromycin B-based reporter strain suitable for simple identification of functional chimeric transcription factor constructs. We showed that the chimeric GaaR-XlnR transcription factor *A. niger* mutant was able to secrete enzymes required for the degradation of pectin when growing on pectin supplemented with D-xylose as inducer compound.

2. Materials and Methods

2.1 Strains, media and growth conditions

Plasmids used in this study were propagated in *Escherichia coli* DH5α, which was grown in Luria-Bertani (LB) medium[25] supplemented with 50 µg/mL ampicillin (Sigma Aldrich, St. Louis, MO, USA). The fungal strains used in this study were derived from *A. niger* CBS 138852 (*cspA1*, *pyrA*$^{-}$, *kusA*::*amdS*)[26], which was obtained from the Westerdijk Fungal Biodiversity Institute culture collection (Utrecht, the Netherlands). All strains generated in this study were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute under accession numbers indicated in Table S1. All fungal strains were maintained by growing at 30°C on *Aspergillus* Minimal Medium (MM) or Complete Medium (CM)[27] supplemented with 1% D-glucose and 1.22 g/L uridine (Sigma Aldrich, St. Louis, MO, USA).

Growth profiles were performed using *Aspergillus* MM with the addition of 25 mM D-glucose, D-galacturonic acid, or D-xylose (Sigma Aldrich, St. Louis, MO, USA) or 1% beechwood xylan, cellulose, xyloglucan, or apple/citrus pectin. All media were supplemented with 1.22 g/L uridine. For antibiotic resistance tests the media were supplemented with 10-25 µg/mL hygromycin B (InvivoGen, San Diego, CA, USA). All growth profile plates were inoculated with 1000 freshly harvested spores and performed in duplicates, and were incubated at 30°C for up to 14 days. Growth was evaluated by visual inspection and pictures were taken at multiple time points.

For liquid cultures, freshly harvested conidia were pre-grown in 250 mL CM containing 2% D-fructose (Sigma Aldrich, St. Louis, MO, USA) and 1.22 g/L uridine for 16 h at 30°C in a rotary shaker at 250 rpm. After 16 h incubation, mycelia were harvested by filtration through sterile cheesecloth, rinsed with MM, and approximately 2.5 g (wet weight) mycelium was transferred in triplicates into 50 mL MM containing 2% wheat

bran, 1% D-xylose, 1% citrus pectin (CP) or 1% citrus pectin supplemented with 0.075% (5 mM) D-xylose (CPX). Supernatant samples were taken after 24 h incubation at 30°C in a rotary shaker at 250 rpm. The samples were centrifuged (10 min, 3220 × *g*, 4°C) and cell-free supernatant samples were stored at -20°C until further processing.

2.2 Construction of mutant strains

CRISPR/Cas9 genome editing was performed using the ANEp8-Cas9-*pyrG* plasmid, which contains the *pyrG* gene as selection marker[24]. The guide RNA (gRNA) sequences were selected by using the Geneious 11.1.4 software (https://www.geneious.com) based on the methodology described by Doench et al.[28]. Repair templates, which include ~750-1000 bp of the 5' and 3' flanking regions of the target sequences, were amplified and fused together using fusion-PCR, and were used to repair the target sequence cleaved by the Cas9 nuclease.

The construction of CRISPR/Cas9 plasmids, generation of *A. niger* protoplasts, transformation and colony purification of putative mutant strains was performed as previously described[29]. The P*pgaX-hph* reporter strain CBS 147359 was generated by replacing the exopolygalacturonase X (*pgaX*) ORF with the hygromycin-B-phosphotransferase (*hph*) ORF originated from *E. coli*[30] in the *A. niger* CBS 138852 background strain. The mutants carrying D-xylose-inducible chimeric GaaR-XlnR constructs were generated by replacing the N-terminal region of XlnR with that of the GaaR in the *A. niger* CBS 138852, CBS 147359 (CBS 138852 P*pgaX-hph*) and CBS 146901 (CBS 138852 Δ*gaaR*) background strains. The constitutively active form of chimeric GaaR-XlnR V756F (corresponding to amino acid mutation V732F in the chimeric sequence) has been generated by simultaneous replacement of XlnR DNA-binding domain and insertion of a point mutation via a single-stranded oligonucleotide in the C-terminal region of XlnR as described before[29].

The generated mutant strains have been confirmed by diagnostic PCR, through the amplification of the target gene region and/or Sanger sequencing (Macrogen Europe, Amsterdam, the Netherlands) (data not shown). For each individual mutation, one candidate was selected for subsequent phenotypic analysis. All primers used in this study were ordered from Integrated DNA Technologies, Inc. (IDT, Leuven, Belgium) and are presented in Table S2.

2.3 In silico analyses

The prediction of coiled coil motifs (Figure S1a, S1b) was performed using the DeepCoil online tool[31] (https://toolkit.tuebingen.mpg.de/tools/deepcoil).

The estimated protein mass was calculated as follows. Signal peptides for secretion were predicted using SignalP 5.0 software tool[32] (http://www.cbs.dtu.dk/services/SignalP/). Estimation of mature amino acid sequence was subsequently calculated using the ProtParam tool from the ExPASy web server (https://web.expasy.org/protparam/).

2.4 SDS-PAGE and enzyme activity assays

Liquid culture filtrates of the control and mutant strains grown in media containing 1% citrus pectin, 1% D-xylose or the combination of 1% citrus pectin and 0.075% (5 mM) D-xylose for 24 h were used to evaluate the produced extracellular CAZymes.

Twelve µL of the culture filtrates were added to 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), incubated at 85°C for 15 min, ice-cooled for 2 min and centrifuged at ~ 10,000 × g for 2 min. Finally, 15 µL of sample were loaded onto 12% (w/v) acrylamide SDS-PAGE gels calibrated with PageRuler prestained protein marker (Thermo Fisher Scientific, Waltham, MA, USA). Visualization was performed by silver staining[33], while documentation was done by using a HP Scanjet G2410 scanner. All samples were evaluated in biological duplicates.

Enzyme activities were performed by using the colorimetric *para*-nitrophenol (*p*NP) or azo-dye substrate assays in 96-well flat bottom microtiter plates. For *p*NP assays, 10 µL supernatant samples were mixed with 10 µL of 0.1% 4-nitrophenyl β-D-glucopyranoside for β-glucosidase (BGL) activity or 4-nitrophenyl β-D-xylopyranoside for β-xylosidase (BXL) activity substrates, 50 µL of 50 mM NaAc (pH 5) and 30 µL of demineralized water in a final volume of 100 µL. Both *p*NP assays were measured after 1 h incubation at 30°C. The reactions were stopped by adding 100 µL of 0.25 M Na_2CO_3 and the absorption values were measured at 405 nm wavelength using a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany). For azo-dye substrate assays 20 µL supernatant samples were mixed with 30 µL of 100 mM NaAc (pH 4.6) and 50 µL of Azo-CM-Cellulose (Megazyme, Bray, Ireland) or Azo-Xylan (birchwood) (Megazyme, Bray, Ireland) substrate for endoglucanase (EGL) and endoxylanase (XLN) activity measurement, respectively. The reaction mixtures were incubated for 4 h at 30°C and

were terminated by the addition of 250 µL of precipitation solution (4% NaAc*3H_2O, 0.4% ZnAc, 76% EtOH, pH 5). The plates were centrifuged at 4°C, 1000 × *g* for 10 min. Supernatant samples were transferred to new microtiter plates and absorption was measured at 600 nm wavelength using a FLUOstar OPTIMA microplate reader. All measurements were performed by using biological duplicates and technical triplicates.

2.5 Saccharification test

Saccharification tests were performed in 96-well flat bottom microtiter plates. Each reaction had 50 mM sodium citrate (pH 5) containing 3% soybean hulls (SBH) or 3% CP mixed with 20 µL culture filtrate in a final volume of 250 µL. The reaction plates were incubated for 6 h at 30°C and 400 rpm. Reactions were stopped by heat inactivation for 15 min at 95°C. The reaction plates were centrifuged for 20 min at 3220 × *g*, and the supernatants were subsequently 10-fold diluted in MilliQ water prior to analysis. The experiment was performed using biological duplicates and technical triplicates.

Monosaccharides were analyzed from peak areas in HPAEC-PAD (Dionex ICS-5000 + system; Thermo Fisher Scientific, Waltham, MA, USA) equipped with CarboPac PA1 column (2×250 mm with 2×50 mm guard column; Thermo Fisher Scientific, Waltham, MA, USA). The column was pre-equilibrated with 18 mM NaOH followed by a multi-step gradient: 0-20 min: 18 mM NaOH, 20-30 min: 0-40 mM NaOH and 0-400 mM sodium acetate, 30-35 min: 40-100 mM NaOH and 400 mM to 1 M sodium acetate, 35-40 min: 100 mM NaOH and 1 M to 0 M sodium acetate followed by re-equilibration of 18 mM NaOH for 10 min (20°C; flow rate: 0.30 mL/min). Concentrations of 5-250 µM of D-xylose, D-galacturonic acid and L-arabinose (Sigma-Aldrich, St. Louis, MO, USA) were used as standards for quantification. Blank samples containing 3% SBH or CP, with the addition of sterile MilliQ water instead of culture filtrates were measured as well. These values were subtracted from each corresponding saccharification sample result in order to exclude the amount of free sugar already present in the experimental condition.

2.6 Proteomics sample preparation and analysis

Proteins from 600 µL cell-free liquid culture filtrates were precipitated by mixing them with two volumes of -20°C methanol, followed by overnight incubation at -20°C.

The precipitated protein solution was centrifuged at 20800 × *g*, 4°C for 20 min. The supernatant was aspirated and the pellet was washed once with 60% cold methanol in water and was resuspended in 6M urea, 100 mM ammonium bicarbonate pH 8 solution. An aliquot was subsequently taken for protein quantification performed colorimetrically using the RCDC kit assay (BioRad, Mississauga, Ontario). In total 7.5 µg of protein samples of biological duplicates were immobilized in acrylamide and processed for in-gel digestion with trypsin as previously described[34]. Dried digest peptide extracts were solubilized in a solution of 5% acetonitrile, 0.1% formic acid and 4 fmol/µL of trypsin-digested Bovine Serum Albumin (BSA) (Michrom, Auburn, CA) used as internal standard. Five µl were analyzed by LC-MS/MS using an Easy-LC II Nano-HPLC system connected in-line with a Velos LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, 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 was performed using the Proteome Discoverer 2.4 (Thermo Fisher Scientific, Waltham, MA, USA) 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. Data analysis was performed based on the percentage values of the total exoproteome.

2.7 Statistical analysis

Statistical analyses were performed on all enzyme assays and saccharification experiments, which were carried out in biological duplicates and technical triplicates. Statistically significant differences (p value < 0.05) were determined using the one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test (Table S3). Analyses were done using STATGRAPHICS Centurion XVI Version 16.1.17 (www.statgraphics.com/centurion-xvi).

3. Results

3.1 The P*pgaX-hph* expression construct allows the screening of functional chimeric transcription factors

The *pgaX* gene encoding an exopolygalacturonase has previously been shown to be under the control of GaaR in *A. niger*[7,9]. Therefore, we selected the promoter of this

gene as a target for screening the activity of GaaR-XlnR chimeric transcription factors, which are able to bind to a GaaR-specific DNA binding site. The CRISPR/Cas9 system was used to delete the ORF of *pgaX*, and a repair template carrying the *hph* gene was used to replace the deleted *pgaX* gene. In order to ensure GaaR-mediated expression of *hph* under the control of *pgaX* promoter (P*pgaX-hph*), the reporter strain was grown on media containing D-galacturonic acid as sole carbon source supplemented with increasing concentrations of hygromycin B. Growth of the parental strain was severely impaired at 10 µg/mL of hygromycin B whereas the reporter strain showed growth when the hygromycin B concentration was in the range of 10-20 µg/mL (Figure 1). However, the reporter strain failed when higher concentrations of hygromycin B were applied. Based on these results, the concentration of 15 µg/mL hygromycin B was used for further screening purposes.

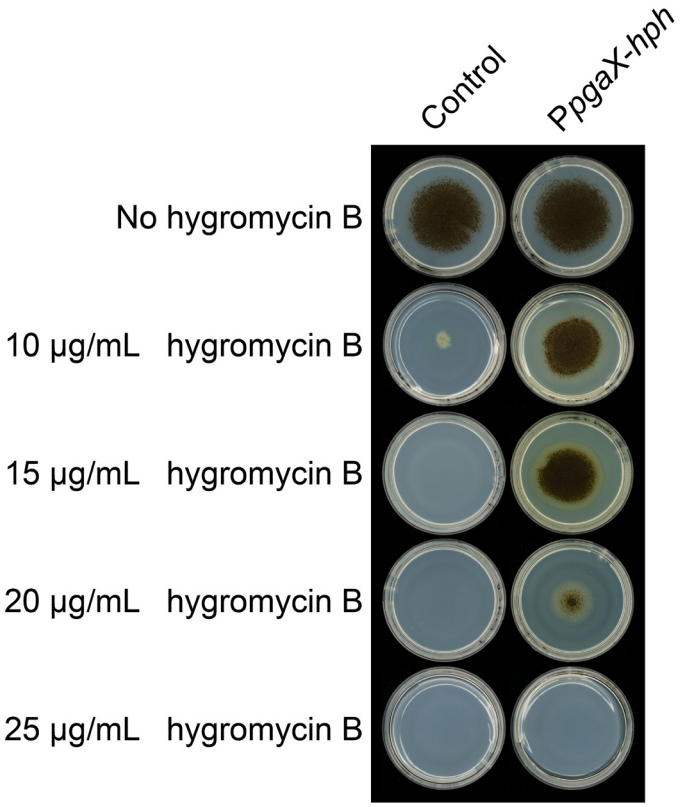


Figure 1. Hygromycin B resistance test of the *A. niger* P*pgaX-hph* reporter strain. The control (CBS 138852) and reporter CBS 147359 (CBS 138852 P*pgaX-hph*) strains were grown on media containing 25 mM D-galacturonic acid as sole carbon source in presence of increasing concentrations (10-25 µg/mL) of antibiotic. Pictures were taken after 10 days of incubation at 30°C.

We constructed three different chimeric GaaR-XlnR models (168.1, 169.1 and 170.1) by fusion PCR to identify an efficient chimera. In all cases the C-terminal region consisted of the amino acids 202-945 of XlnR, which includes the fungal transcription factor activation domain[35]. The three constructs differ in the GaaR N-terminal regions, which were selected based on prediction of putative coiled-coil elements (Figure S1a) and amino-acid sequence conservation across a wide range of filamentous fungi (data not shown). In case of the chimeric model 168.1, the N-terminal 1-107 amino acid sequence of GaaR was fused together with the C-terminal region of XlnR (Figure S1c). The N-terminal sequence of GaaR retained its endogenous zinc-finger domain followed by a linker and a hypothetical coiled-coil sequence, as in silico predicted. For model 169.1 the N-terminal GaaR region consisted of the amino acids 1-193, carrying an additional putative coiled-coil region (Figure S1d); while in model 170.1 the GaaR fragment consisted of a larger N-terminal fragment of 1-229 amino acids (Figure S1e). The expression of each chimeric construct was driven by the endogenous *xlnR* promoter. The chimeric GaaR-XlnR mutants were generated in the P*pgaX-hph* background strain to assess the function of the chimeric constructs.

Subsequently, the control (CBS 138852), P*pgaX-hph* and mutants carrying each of the three chimeric GaaR-XlnR constructs in a P*pgaX-hph* background were tested for growth on media containing D-galacturonic acid or D-xylose supplemented with 15 µg/mL hygromycin B (Figure 2). None of the strains showed reduced growth on the media containing 25 mM D-glucose, 25 mM D-galacturonic acid or 25 mM D-xylose in the absence of hygromycin B. These results suggest that the tested strains do not display metabolic defects in the utilization of D-glucose, D-galacturonic acid and D-xylose; and that the differential growth observed on media supplied with 15 µg/mL hygromycin B is attributed to the presence of the drug. The P*pgaX-hph* reporter strain, as well as the chimeric mutants showed substantial growth on D-galacturonic acid supplied with hygromycin B. When D-xylose was the sole carbon source, the chimeric mutants 169.1 and 170.1 showed growth comparable to that of the reporter strain after 8 days of incubation, suggesting insufficient expression of the reporter construct mediated through their chimeric GaaR-XlnR transcription factors. In contrast, the chimeric mutant 168.1 showed substantially improved growth and sporulation compared to the reporter strain and the other chimeric mutants. Thus, the chimeric model 168.1 was chosen for further characterization.

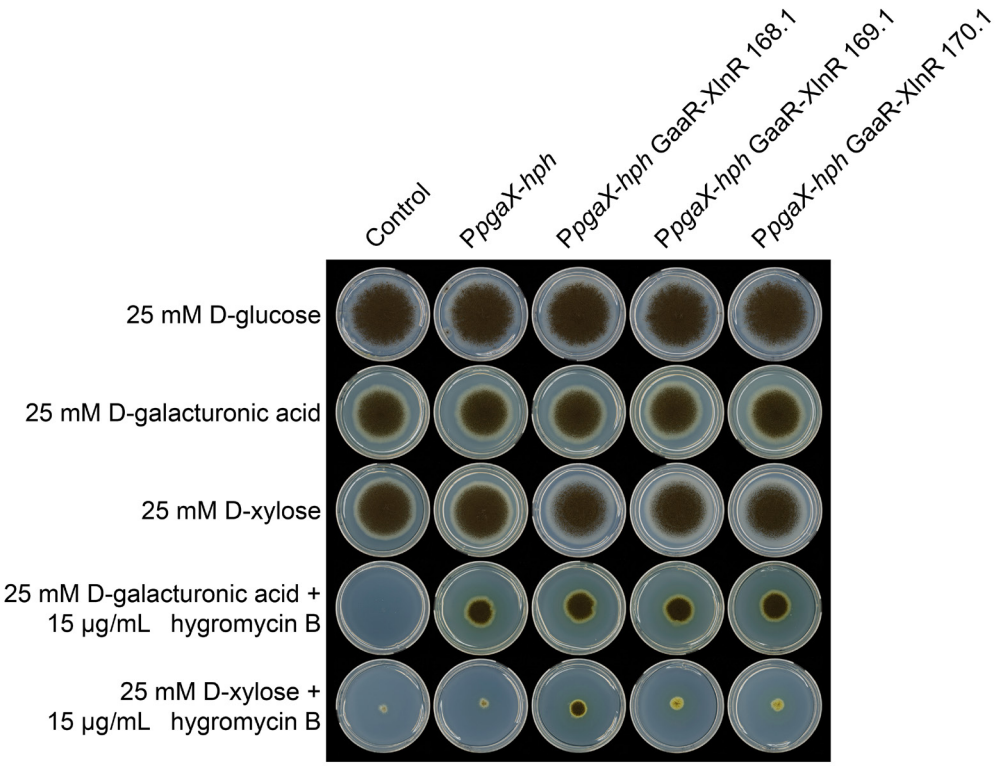


Figure 2. Functionality test of GaaR-XlnR chimeric transcription factor mutants. The growth of control (CBS 138852), P*pgaX-hph* reporter strain and the P*pgaX-hph* strains carrying chimeric GaaR-XlnR constructs was tested on MM containing either 25 mM D-xylose or D-galacturonic acid as carbon source supplemented with 15 µg/mL hygromycin B. Plates without hygromycin B and with D-glucose as the sole carbon source were included as reference. Pictures were taken after 8 days of incubation at 30°C.

3.2 The chimeric GaaR-XlnR mutant showed impaired XlnR activity

Based on our initial screening, the GaaR-XlnR chimeric model 168.1 was used to generate a GaaR-XlnR chimeric mutant in the *A. niger* CBS 138852 background strain. Moreover, the CRISPR/Cas9 system was also used to generate a constitutively active form of the chimeric transcription factor by introducing a point mutation (V756F) in the C-terminal region of XlnR as described before[29]. XlnR has been described to be regulated on a post-translational level through a proposed D-glucose inhibitory domain found in its C-terminal region, which is responsible for turning XlnR into an inactive state under repressing conditions[35]. The V756F mutation disturbs this inhibitory domain, keeping

XlnR in a permanently active form[35]. Therefore, the constitutively active form of the chimeric transcription factor is independent of the presence of the activator (D-xylose), showing a clearer phenotype of the chimeric mutation. Growth profile results showed that the growth of the GaaR-XlnR and the GaaR-XlnR V756F mutants was comparable to that of the Δ*xlnR* mutant (Figure 3a), indicating that the replacement of the XlnR N-terminal region for that of GaaR resulted in the loss of native XlnR function. The extracellular protein profile of these mutants grown on 2% wheat bran liquid cultures further supported this observation, since the production of the major endoxylanases found in the molecular mass range of 13-33 kDa[36] was highly reduced (Figure 3b). Moreover, enzyme activity assays (Figure 3c) confirmed the abolition of beta-xylosidase (BXL) activity, and the high reduction in endoxylanase activity (XLN) (Figure 3c), which are both required for the efficient degradation of xylan and are (mainly) under the control of XlnR. The impaired growth on cellulose (Figure 3a) also correlates with the overall reduction of cellulolytic activities, indicated by the beta-glucosidase (BGL) and endoglucanase (EGL) activities (Figure 3c).

3.3 The GaaR-XlnR chimeric transcription factor recovers growth on pectin in a Δ*gaaR* strain

To evaluate the pectinolytic activities mediated by a GaaR-XlnR chimeric transcription factor, the GaaR-XlnR and GaaR-XlnR V756F mutations were generated in a Δ*gaaR* background strain. Growth profiling (Figure 4) showed abolished growth on D-galacturonic acid, and highly reduced growth on apple and citrus pectin for Δ*gaaR*. The Δ*gaaR* GaaR-XlnR mutant showed comparable growth to Δ*gaaR* on 25 mM D-galacturonic acid, as well as on 1% apple pectin or 1% citrus pectin. However, the addition of 2 mM D-xylose resulted in substantial growth recovery on these substrates. In contrast, Δ*gaaR* GaaR-XlnR V756F improved growth compared to Δ*gaaR* even without the addition of 2 mM D-xylose, thus demonstrating the inducer-independent nature of this chimeric transcription factor. All strains showed minimal growth on 2 mM D-xylose as sole carbon source, indicating that the differential growth of chimeric mutants on substrates with or without 2 mM D-xylose is not attributed to the metabolism of D-xylose present in the media.

Despite the addition of D-xylose, none of the chimeric mutants showed fully recovered growth on 25 mM D-galacturonic acid.

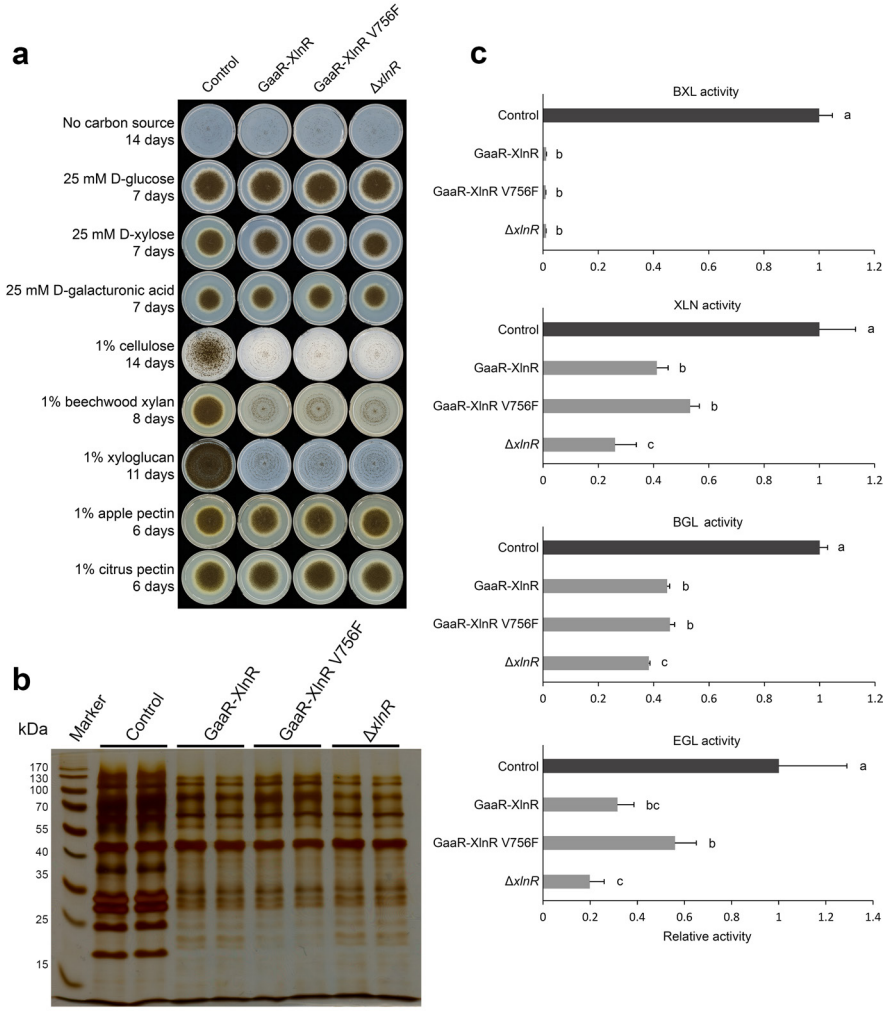


Figure 3. Phenotypic screening of GaaR-XlnR chimeric mutant strains. a) Growth profile of *A. niger* control (CBS 138852), GaaR-XlnR, GaaR-XlnR V756F and Δ*xlnR* strains on selected mono- and polysaccharides. All plates were incubated at 30°C for up to 14 days. Note that the Δ*xlnR* strain was included as a negative control for the loss of XlnR function. **b**) Extracellular protein analysis of *A. niger* control (CBS 138852) and mutant strains. Supernatant filtrates were harvested from 2% wheat bran liquid cultures incubated at 30°C and 250 rpm for 24 h. **c**) Enzyme activity assays of supernatant filtrates originated from 2% wheat bran liquid cultures after 24 h incubation at 30°C and 250 rpm. Graph bars represent normalized enzyme activity values, and letters (a-c) represent the statistical differences between samples within each specific enzyme assay. Samples showing different letters show significant differences among the strains, while samples sharing the same letters show no statistically significant differences (ANOVA and Tukey's HDS test, $p < 0.05$).

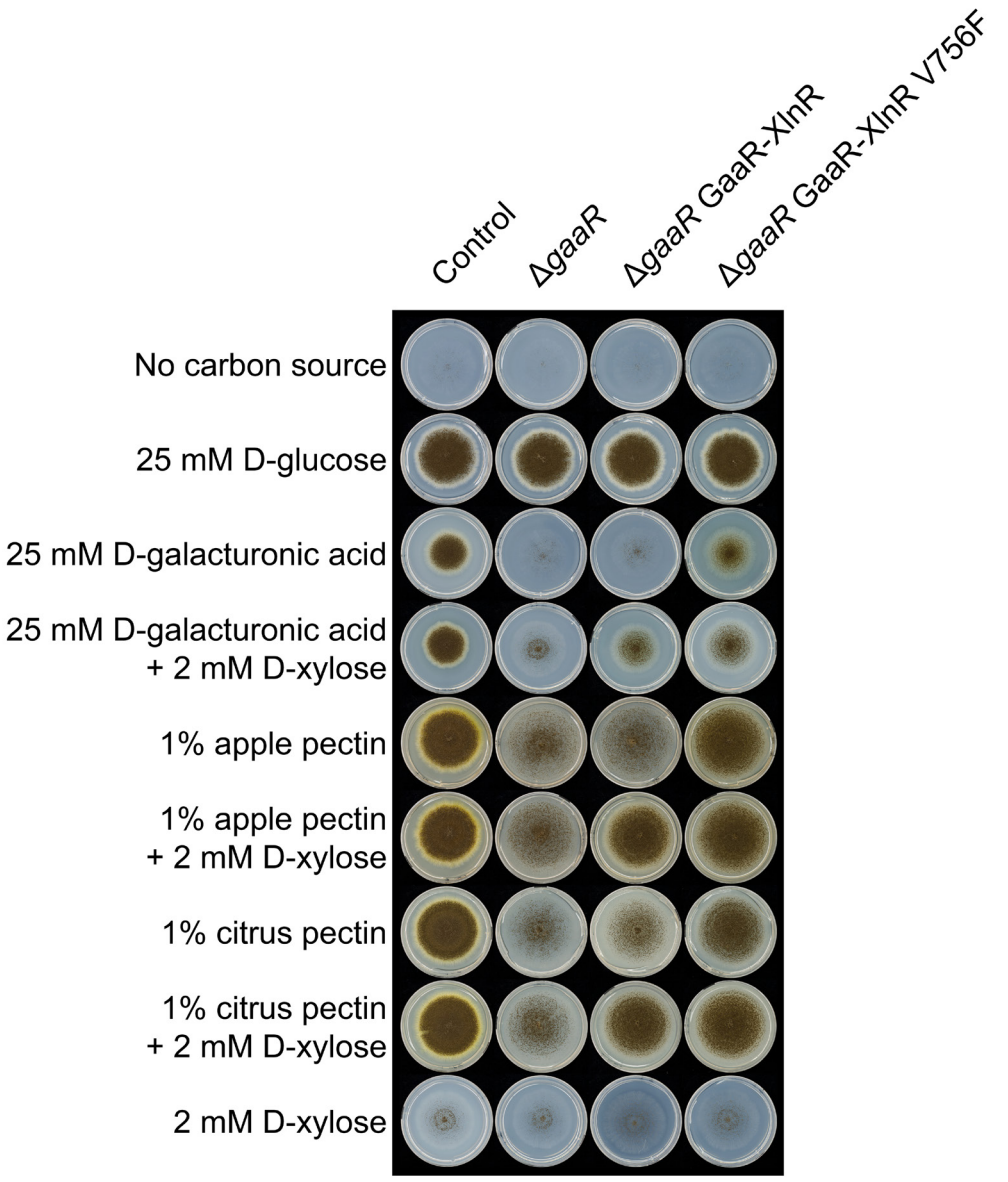


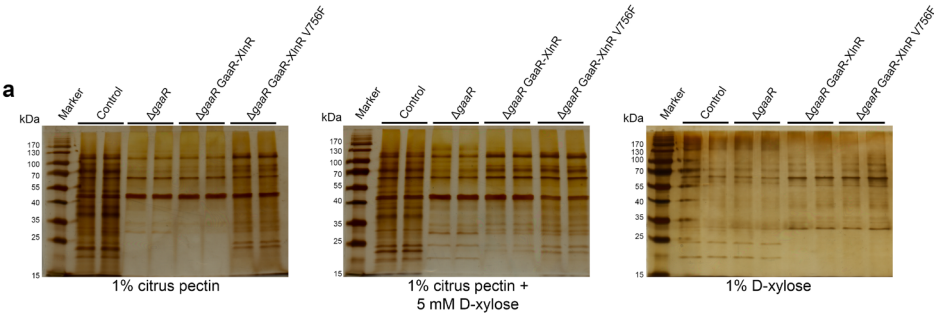
Figure 4. Growth test of GaaR-XlnR chimeric mutants on pectin and related substrates. The control (CBS 138852), Δ*gaaR*, Δ*gaaR* GaaR-XlnR and Δ*gaaR* GaaR-XlnR V756F strains were grown on media containing 1% apple/citrus pectin and D-galacturonic acid. The tests substrates were also supplemented with D-xylose for the induction of the chimeric transcription factor. All plates were incubated for 7 days at 30°C.

3.4 Chimeric GaaR-XlnR transcription factor activates the production of pectinolytic enzymes

To evaluate which pectinolytic proteins are produced by the chimeric GaaR-XlnR mutants, the *gaaR* deficient mutants carrying the chimeric transcription factor were cultivated in 1% citrus pectin and 1% citrus pectin + 5 mM D-xylose liquid media. SDS-PAGE analysis showed comparable protein production for Δ*gaaR* and Δ*gaaR* GaaR-XlnR after 24h incubation in 1% citrus pectin (Figure 5a). In contrast, Δ*gaaR* GaaR-XlnR V756F showed substantially improved protein production compared to both Δ*gaaR* and Δ*gaaR* GaaR-XlnR. However, the protein pattern of the mutant carrying the constitutively active chimeric transcription factor did not show complete recovery of extracellular enzymes compared to the control (CBS 138852) strain. When cultivated on 1% citrus pectin + 5 mM D-xylose or on 1% D-xylose both mutants carrying the chimeric GaaR-XlnR construct showed a comparable extracellular protein profile (Figure 5a). This result correlates with the observed growth phenotype (Figure 4), indicating the induction of the chimeric GaaR-XlnR in the presence of D-xylose and the inducer independent activity of the constitutively active GaaR-XlnR V756F form.

Proteomic analysis showed partial or full recovery of the production of several proteins involved in pectin degradation in Δ*gaaR* GaaR-XlnR V756F compared to Δ*gaaR* (Figure 5b) when cultured on citrus pectin (CP) and citrus pectin supplemented with D-xylose (CPX). Several proteins (e.g., NRRL3_5252, RgaeB, PgxB and GalA) increased in levels in both conditions (CP and CPX) in the constitutively active mutant. However, the addition of D-xylose to the culture media did not result in increased production of these proteins in Δ*gaaR* GaaR-XlnR. In contrast, an endo-polygalacturonase (PgaB), an endo-1,6-beta-galactanase (NRRL3_8701) and two α-L-arabinofuranosidases (AbfA and AbfB) showed substantially increased abundance in both CP and CPX filtrates of Δ*gaaR* GaaR-XlnR V756F, as well as in the CPX filtrate of Δ*gaaR* GaaR-XlnR. The arabinofuranosidase AbfB showed the highest abundance in the exoproteome of both Δ*gaaR* GaaR-XlnR and Δ*gaaR* GaaR-XlnR V756F filtrates. Interestingly, both the cellobiohydrolase CbhB and the glucoamylase GlaA showed upregulation in the CPX filtrate exoproteome of both chimeric mutants compared to Δ*gaaR* (Supplementary Data 1). These proteins were also the most abundant ones in the exoproteome next to AbfB.

a



b

Gene number	Gene name	Activity/description	CAZy family	Estimated molecular weight (kDa)	Control CP	Δ*gaaR* CP	Δ*gaaR* GaaR-XlnR CP	Δ*gaaR* GaaR-XlnR V756F CP	Control CPX	Δ*gaaR* CPX	Δ*gaaR* GaaR-XlnR CPX	Δ*gaaR* GaaR-XlnR V756F CPX
NRRL3_3768	*abfB*	ABF (α-arabinofuranosidase)	GH54	50.66	5.77	4.68	2.78	9.68	12.86	1.99	5.13*	16.00
NRRL3_5252	-	PME (pectin methyl esterase)	CE8	41.33	8.51	0.00	0.00	0.48	8.05	0.00	0.02	0.51
NRRL3_5407	*abnC*	ABN (endo-arabinanase)	GH43	32.21	0.08	0.42	0.44	0.42	0.13	0.75	0.29	0.62
NRRL3_7501	*rgaeB*	RGAE (rhamnogalacturonan acetyl esterase)	CE12	26.20	1.56	0.04	0.04	0.33	0.37	0.04	0.05	0.27
NRRL3_8281	*pgxB*	PGX (exo-polygalacturonase)	GH28	46.79	2.25	0.00	0.00	0.29	1.30	0.00	0.00	0.27
NRRL3_2630	*lacA*	LAC (β-1,4-galactosidase)	GH35	108.04	0.41	0.82	0.20	0.27	0.05	0.68	0.77	0.53
NRRL3_5859	*pgaB*	PGA (endo-polygalacturonase)	GH28	35.88	0.93	0.15	0.10	0.26	0.34	0.09	0.24*	0.39
NRRL3_8701	-	GLN (exo-1,6-galactanase)	GH5_16	43.59	0.15	0.08	0.06	0.16	0.02	0.06	0.31*	0.92
NRRL3_7469	*xghA*	XGH (xylogalacturonase)	GH28	40.20	2.35	0.08	0.06	0.16	1.03	0.01	0.05	0.08
NRRL3_10643	*galA*	GAL (β-1,4-endo-galactanase)	GH53	37.05	0.61	0.00	0.00	0.14	0.90	0.00	0.00	0.11
NRRL3_965	*pelA*	PEL (pectin lyase)	PL1_4	37.85	0.23	0.00	0.01	0.13	0.09	0.02	0.00	0.06
NRRL3_1606	*abfA*	ABF (α-arabinofuranosidase)	GH51	65.38	0.24	0.05	0.07	0.12	0.03	0.16	0.31*	0.46
NRRL3_8325	*pmeA*	PME (pectin methyl esterase)	CE8	33.93	0.42	0.00	0.00	0.04	0.43	0.00	0.00	0.02
NRRL3_3144	*pgaX*	PGX (exo-polygalacturonase)	GH28	45.04	1.90	0.00	0.00	0.00	0.29	0.00	0.00	0.00
NRRL3_10559	*rgxC*	RGX (exo-rhamnogalacturonase)	GH28	43.85	1.31	0.00	0.00	0.00	0.13	0.00	0.00	0.00
NRRL3_8805	*pgaC*	PGA (endo-polygalacturonase)	GH28	38.57	0.64	0.00	0.00	0.00	0.02	0.00	0.00	0.00
NRRL3_2835	*pgaE*	PGA (endo-polygalacturonase)	GH28	37.70	0.46	0.00	0.00	0.00	0.07	0.00	0.00	0.00
NRRL3_7470	*pmeB*	PME (pectin methyl esterase)	CE8	32.81	0.42	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NRRL3_2571	*pgaI*	PGA (endo-polygalacturonase)	GH28	36.31	0.33	0.00	0.00	0.00	0.01	0.00	0.00	0.00
NRRL3_9126	*rhgA*	RHG (endo-rhamnogalacturonase)	GH28	45.22	0.29	0.00	0.00	0.00	0.14	0.00	0.00	0.03
NRRL3_5260	*pgxC*	PGX (exo-polygalacturonase)	GH28	45.76	0.14	0.00	0.00	0.00	0.08	0.00	0.00	0.00
NRRL3_10115	-	RGL (rhamnogalacturonan lyase)	PL4_3	71.78	0.03	0.23	0.13	0.00	0.00	0.59	0.46	0.05
NRRL3_3279	-	RHA (α-rhamnosidase)	GH78	89.73	0.01	0.19	0.33	0.00	0.00	0.21	0.14	0.05
NRRL3_2162	-	RHA (α-rhamnosidase)	GH78	68.54	0.00	1.03	0.79	0.00	0.00	0.95	0.11	0.00
NRRL3_7	*faeA*	FAE (feruloyl esterase)	esterase	28.36	0.00	0.28	0.03	0.00	0.00	0.20	0.00	0.00

Figure 5. Exoproteome analysis of GaaR-XlnR chimeric mutants in Δ*gaaR* background strain. **a**) SDS-PAGE analysis of supernatant samples. Supernatant filtrates of control (CBS 138852), Δ*gaaR*, as well as Δ*gaaR* GaaR-XlnR and Δ*gaaR* GaaR-XlnR V756F chimeric transcription factor mutants were harvested after 24 h incubation at 30°C and 250 rpm. Different liquid culture conditions are indicated in the figure. Samples are shown as biological duplicates. **b**) Selected proteins associated with the degradation of pectin detected by proteomics analysis in the exoproteome of control (CBS 138852) and mutant strains. Samples were harvested from 1% citrus pectin (CP) or 1% citrus pectin + 5 mM D-xylose (CPX) liquid cultures. Protein abundance is represented as percentage of the total exoproteome. Proteins with an abundance < 0.1% of the total exoproteome are indicated in grey cells. Proteins showing > 1.5-fold increase in the chimeric mutants compared to the Δ*gaaR* strain are indicated in green cells. Asterisk (*) indicates the proteins which showed increased abundance compared to the Δ*gaaR* strain only when the media was supplemented with D-xylose. The total exoproteome data are found in Supplementary Data 1.

3.5 The GaaR-XlnR V756F chimeric transcription factor improves the release of D-galacturonic acid and L-arabinose from pectin

The saccharification and sugar release analysis of 3% soybean hulls (SBH) and 3% CP was performed to evaluate the enzymatic activities present in the CP and CPX liquid

culture filtrates of the control (CBS 138852) and mutant strains after 24 h incubation. Soybean hulls has been selected as a crude substrate for saccharification due to its high pectin content represented by the abundant presence of D-galactose, L-arabinose and D-galacturonic acid in its composition[37]. The amount of released D-xylose from SBH was low (< 0.1 mM) for all strains, including the control (Figure S2). However, no D-xylose has been detected when either the filtrate of Δ*gaaR* GaaR-XlnR or Δ*gaaR* GaaR-XlnR V756F was used. None of the filtrates resulted in the release of D-xylose from 3% CP, most likely due to the low abundance of this sugar in its composition. Surprisingly, the release of D-galacturonic acid from either 3% SBH (Figure 6a) or 3% CP (Figure 6b) did not improve in case of Δ*gaaR* GaaR-XlnR compared to Δ*gaaR* at any of the conditions tested. In contrast, Δ*gaaR* GaaR-XlnR V756F showed consistently improved D-galacturonic acid release when either the CP or CPX filtrates were used (Figure 6a, b). However, this amount was lower than that of the control strain. On the other hand, the release of L-arabinose from 3% SBH (Figure 6c) and 3% CP (Figure 6d) was comparable for all mutant strains when the CP filtrates were used, with a slight improvement of release in case of the constitutive GaaR-XlnR V756F from 3% CP (Figure 6d). The chimeric mutants showed the most significant improvement of L-arabinose release compared to Δ*gaaR* when the CPX filtrates were used.

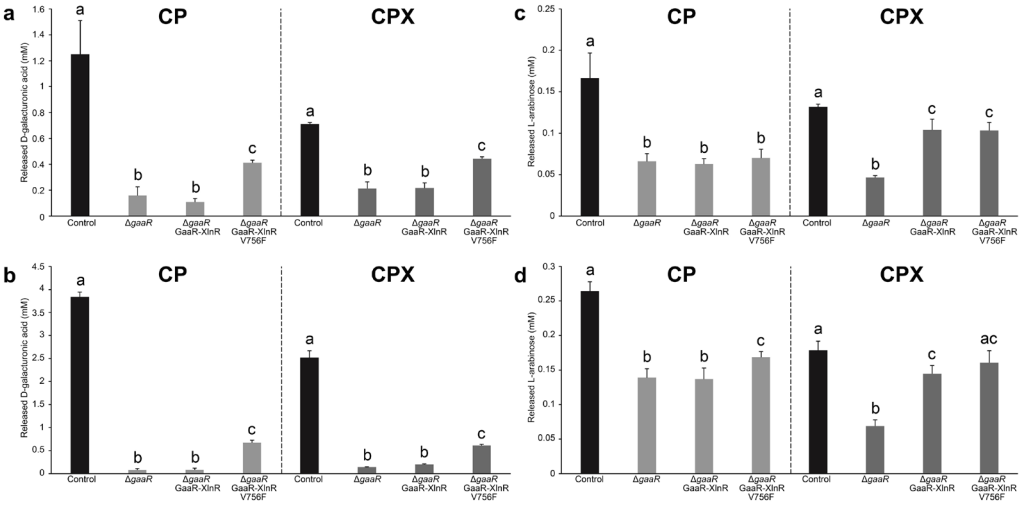


Figure 6. Saccharification analysis of 3% soybean hulls and 3% citrus pectin substrates by *A. niger* control (CBS 138852) and mutant strain supernatant filtrates. Supernatant filtrates were harvested from 1% citrus pectin (CP) or 1% citrus pectin + 5 mM D-xylose (CPX) liquid cultures. Graphs illustrate the amount of released D-galacturonic acid from 3% soybean hulls (**a**) and 3% citrus pectin (**b**), as well as the released L-arabinose from 3% soybean hulls (**c**) and 3% citrus pectin (**d**). Letters (a-c) represent the statistical differences between samples within each specific saccharification assay. Samples showing different letters show significant differences among the strains, while samples sharing the same letters show no statistically significant differences (ANOVA and Tukey's HDS test, $p < 0.05$).

4. Discussion

In this study, we tested three versions of GaaR-XlnR chimeric transcription factor mutants to generate an *A. niger* strain that is able to produce pectinolytic enzymes by using D-xylose as inducing sugar.

The majority of fungal transcriptional activators belong to the Zn_2Cys_6 cluster family proteins. These proteins usually carry the DNA binding motif at the N-terminal region, while the activation domain is found in the C-terminal region of the protein[38]. It has been reported that the binding specificity of a Zn_2Cys_6 transcription factor is affected by the linker region on the C-terminal end of the zinc-finger motif[10,39]. However, it has also been shown that binding specificity can be influenced by the N-terminal end of a putative dimerization element represented by a coiled-coil following the linker region[11]. Considering these observations, we decided to fuse the C-terminal region of XlnR (202-945 aa) together with three different N-terminal GaaR fragments, all of them including the zinc-finger motif (26-53 aa), linker region (54-64 aa) and the neighboring putative coiled-coil element (65-85 aa) (Figure S1).

To simplify the screening of chimeric GaaR-XlnR constructs, we constructed a P*pgaX-hph* reporter strain. The utilization of the endogenous *pgaX* promoter-reporter system for the analysis of GaaR-mediated activation of a selection marker gene (*amdS*) has previously been reported[40]. In our study, we used the hygromycin B phosphotransferase gene (*hph*) for screening purposes. It has been reported that *A. niger* is able to grow when the concentration of hygromycin B is even higher than 100 µg/mL[41]. However, in most cases the *hph* gene is expressed under the control of a strong constitutive promoter, such as P*gpdA* from *Aspergillus nidulans*. Initial screening indicated that the GaaR (1-107 aa)-XlnR (202-945 aa) chimera (model 168.1) showed the best resistance to hygromycin B when induced by D-xylose (Figure 2), indicating that the presence of additional GaaR sequence elements (models 169.1, 170.1) might result in the loss of function and/or reduced stability of the chimeric transcription factor. However, the slow growth of this mutant indicates that the chimeric transcription factor only partially activates the expression of the reporter gene compared to the endogenous GaaR. This result correlates with the observation that certain chimeric constructs show relatively low affinity towards some specific target genes[12].

Phenotypic analysis indicated the high reduction or even abolition of enzymatic activities affected by XlnR in the GaaR-XlnR and GaaR-XlnR V756F mutants (Figure 3). Although the XLN, BGL and EGL enzyme activities were highly reduced, they were not completely abolished in any of the chimeric mutants, most likely due to the involvement of other

(hemi-)cellulolytic transcription factors, such as ClrA and/or ClrB in the regulation of these activities in *A. niger*[42]. However, the residual cellulolytic activities were not sufficient to support growth on cellulose (Figure 3a). Interestingly, the XLN, BGL and EGL activities were in general slightly higher than in the Δ*xlnR* strain, which might indicate residual direct or indirect activation of the corresponding genes. The growth on pectin was not increased in the chimeric mutant strains compared to the control, suggesting that the chimeric transcription factor does not improve the expression of pectinolytic genes in the presence of an active GaaR. However, the activation of the pectinolytic system by the chimeric transcription factor was more prominent when the chimeric mutation was introduced in a Δ*gaaR* background strain. The addition of 2 mM D-xylose to the growth test substrates clearly shows the activation of the GaaR-XlnR chimeric transcription factor, which subsequently resulted in a substantial growth recovery on pectin. The chimeric mutant was able to grow on D-galacturonic acid after induction with D-xylose, but growth was only partially recovered. This indicates that the chimeric transcription factor is also able to activate all the essential genes encoding for the D-galacturonic acid catabolism. However, the artificial transcription factor might not have sufficient expression level or might not be stable enough to maintain a metabolic flux similar to the wild type. One alternative to improve the overall activity of the chimeric transcription factor would be the utilization of a strong constitutive promoter, as done in previous studies for the expression of other artificial transcription factors[13,15,18,19], resulting in higher enzymatic activities.

The exoproteome patterns (Figure 5a) of Δ*gaaR* GaaR-XlnR and Δ*gaaR* GaaR-XlnR V756F correlate with the growth profile results (Figure 4), indicating that the constitutively active version of the chimeric transcription factor can activate its target genes in the absence of the inducing sugar. Proteomics analysis confirmed the activation of pectinolytic genes by the GaaR-XlnR chimeric transcription factor, showing substantially increased abundance of pectinolytic enzymes in the constitutively active Δ*gaaR* GaaR-XlnR V756F mutant compared to Δ*gaaR*. Of the proteins that showed increased abundance by Δ*gaaR* GaaR-XlnR in the CPX condition, AbfA[7,9] and PgaB[9] have previously been described to be controlled by GaaR. The low abundance of AbfB in Δ*gaaR* compared to the control and the substantially increased abundance in the chimeric transcription factor mutants in the CPX condition indicates that production of this enzyme is also dependent on GaaR mediated-induction of the corresponding gene. Although GaaR is responsible for the production of most enzymes involved in the degradation of the polygalacturonic acid backbone[9], in our experimental conditions, especially in the CPX medium, the expression of arabinanolytic genes was the most

prominent. The molecular weight of the pectinolytic proteins found in the supernatants of the chimeric mutants have been estimated in this study (Figure 5b), but due to post-translational modifications such as glycosylation, the estimated molecular weight of the proteins detected by proteomics analysis and the exoproteome pattern observed by SDS-PAGE analysis cannot be directly correlated. However, the molecular weight of the α-L-arabinofuranosidases AbfA and AbfB have been experimentally determined to be 83 kDa and 67 kDa, respectively[43], which appear at a higher intensity on the SDS-PAGE profile of the chimeric mutants when D-xylose is present in the liquid media (Figure 5a).

The saccharification results also support the activation of arabinofuranosidases in the chimeric mutants. Interestingly, both CbhB and GlaA showed significant increase in abundance in the chimeric mutants, which is most likely an indirect effect of the chimeric mutation.

In conclusion, in this study we report the utilization of CRISPR/Cas9 genome editing to generate a GaaR-XlnR chimeric transcription factor by precise on-site mutagenesis in *A. niger* for the first time. This artificial transcription factor was able to recover lost GaaR functions when induced by D-xylose. Moreover, the alteration of the specificity of the endogenous XlnR resulted in the downregulation of several (hemi-)cellulolytic enzymes due to the loss of XlnR activity, which can reduce the purification costs of pectinase-rich enzyme cocktails. Even though the chimeric mutant showed the upregulation of several pectinolytic proteins compared to the Δ*gaaR* strain, the abundance was in general lower than that of the control strain. These results may indicate that the expression level or the stability of the chimeric transcription factor could be improved. The utilization of a strong constitutive promoter for the expression of GaaR-XlnR might further improve the expression level of this artificial transcription factor and could possibly result in enriched pectinolytic enzyme cocktails when grown on D-xylose or xylan-rich substrates, with all the benefits that this would entail at the biotechnological and industrial level.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[44] partner repository with the dataset identifier PXD025383 and 10.6019/PXD025383 (http://www.ebi.ac.uk/pride/archive/projects/PXD025383).

Author contribution

RSK performed the experiments, analyzed data, and wrote the original manuscript. SG contributed to data analysis and reviewed and edited the manuscript. MDF performed the proteomics analysis and reviewed and edited the manuscript. AT and RPdV designed the experiments, supervised the research, and reviewed and edited the manuscript.

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Supporting information

All supporting information are available at: https://doi.org/10.1007/s00253-021-11428-2 or can be obtained upon request from the author.

Figure S1. Sequence analysis of *A. niger* GaaR and XlnR transcription factors. a, b) Prediction of putative coiled-coil regions in GaaR (**a**) and XlnR (**b**) amino acid sequence. Vertical black lines indicate the approximate position of the terminal amino acid of the N-terminal region of GaaR (**a**) and the starting amino acid of the C-terminal region of XlnR (**b**). **c-e** Amino acid sequence of chimeric GaaR-XlnR transcription factor model 168.1 (**c**), 169.1 (**d**) and 170.1 (**e**). The zinc-finger domain is indicated in green letters and the linker region in bold letters. The predicted coiled coil, which is putatively responsible for dimerization is indicated with underlined letters. The 202-945 aa C-terminal region of XlnR is indicated in red.

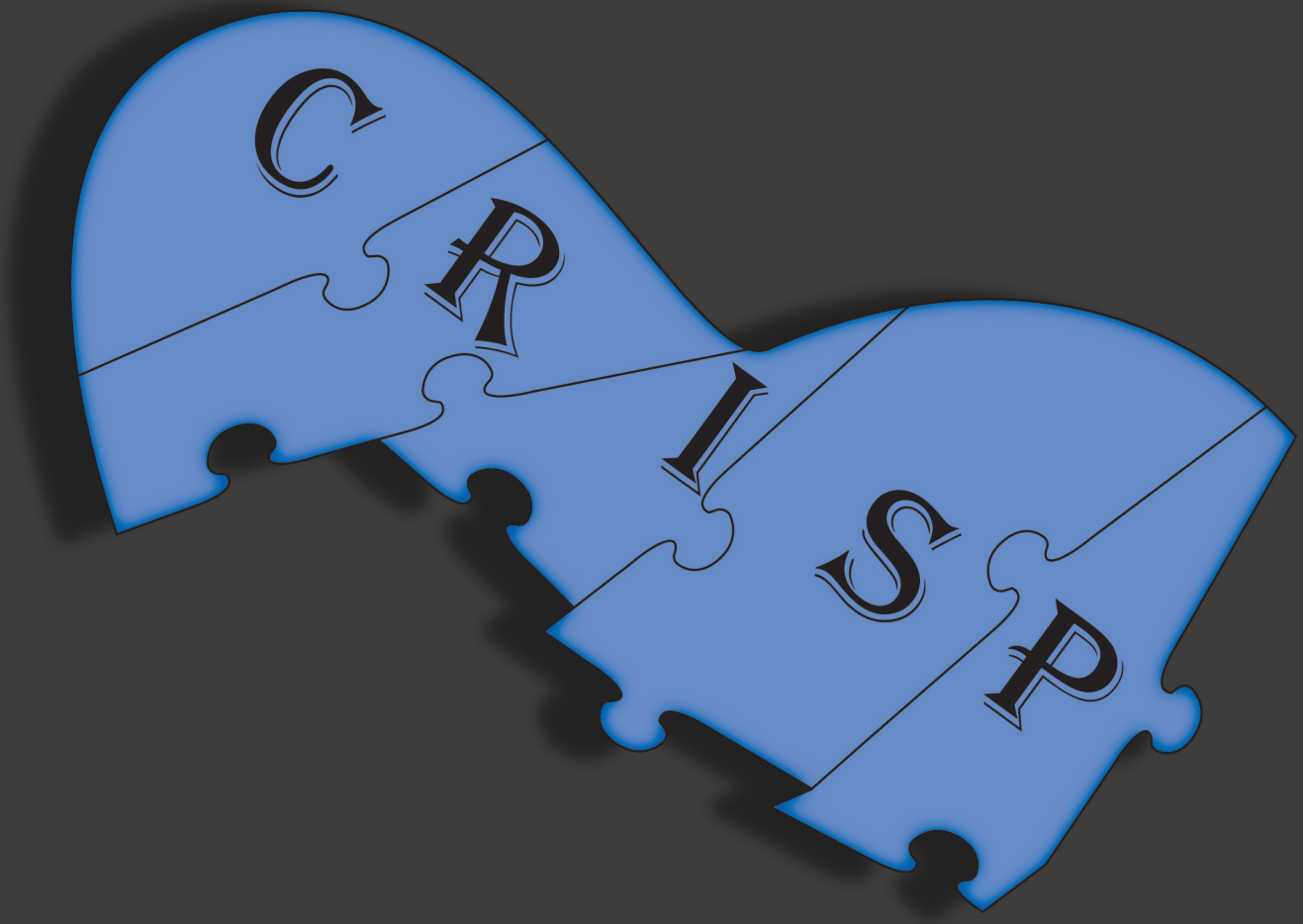
Figure S2. D-xylose released from 3% soybean hulls by *A. niger* control (CBS 138852) and mutant strains. Supernatant filtrates of tested strains originated from 1% citrus pectin (CP) or 1% citrus pectin + 5 mM D-xylose (CPX) liquid cultures.

Table S1. *Aspergillus niger* strains used in this study.

Table S2. Primers used in this study. Homology flanks are highlighted in red.

Table S3. Summary of the ANOVA analysis for each enzyme assay and saccharification test.

Supplementary Data 1. Proteomics results of *A. niger* control (CBS 138852) and Δ*gaaR*, Δ*gaaR* GaaR-XlnR and Δ*gaaR* GaaR-XlnR V756F mutant strains. Supernatant filtrates originated from 1% citrus pectin (CP) or 1% citrus pectin supplemented with 5 mM D-xylose (CPX). Protein percentage values, which are < 0.1% are highlighted in grey cells.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Chapter 5

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[1]. One such substrate is guar gum (GG), an abundant biopolymer found in *Cyamopsis tetragonolobus* seed endosperm[2], which is used as a thickener, stabilizer agent and additive in pharmaceutical, paper, textile or food industries[3]. Its main component is galactomannan[4], a polymer which is also abundantly present in soybean hulls (SBH)[5]. 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[6]. Soybean hulls are used as animal feed, in the treatment of waste water or as dietary fibers[6].

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, www.cazy.org)[7] they can secrete. Transcription Factors (TF) play an essential role in the regulation of gene expression, controlling the production of a set 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[8]. Some transcription factors are associated with the degradation of specific substrates, such as GaaR[9], which is the key regulator of pectin degradation[10]. Other TFs control the degradation of a broader set substrates. For instance, XlnR[11] controls the expression of cellulolytic, xylanolytic, mannanolytic and xyloglucanolytic genes[12–14], while AraR[15] plays an important role in hemicellulose and pectin degradation[10].

In *N. crassa*, the regulation of cellulose degradation is controlled by two TFs, CLR-1 and CLR-2[16]. ClrA, the ortholog of CLR-1, has also been identified in *Aspergillus nidulans*[16] and *A. niger*[17,18] and was reported to be involved in cellulose degradation in these species. Orthologs of CLR-2 have been identified in *A. nidulans* (ClrB)[16,19], *A. niger* (ClrB)[17], *A. oryzae* (ManR)[20] and *Penicillium oxalicum* (ClrB)[21] that showed conserved function with respect to cellulose degradation, although *A. oryzae* ClrB (ManR) had been first reported as a TF regulating mannan degradation[20]. A later study also showed the involvement of *N. crassa* CLR-2 in the regulation of mannan utilization[22]. 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*[23]. However, it was reported that the induction of mannanases

in *A. nidulans* is more likely controlled by a ClrB paralogue, AN6832[23], which is not present in *A. oryzae* or *A. niger*[24]. The induction and/or the role of CLR-2/ClrB/ManR orthologs within the regulatory network of (hemi-)cellulose degradation appears strikingly different[16,19,24]. 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[25]. A less conserved homolog of ClrB (CLR-2) has also been described in *Trichoderma reesei*, but results showed only a minor influence of CLR-2 on cellulase regulation in this species[26]. In contrast, light-dependent control of xylanase and pectinase encoding genes has been associated with CLR-2 in *T. reesei*[27]. Even though the involvement of *A. niger* ClrB in cellulose utilization has been previously reported[17,18], it is not fully known to which extent it may also control the degradation of mannan or other plant polysaccharides.

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[28]. All deletion mutants used in this study were previously generated by using the CRISPR/Cas9 system[18] 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)[29] 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[18]. 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[30].

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[30]. 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[31]. 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[32] 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[33] (http://rsat-tagc.univ-mrs.fr/rsat/dna-pattern_form.cgi) as previously reported[18]. 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 (Figure 1). GG is mainly composed of galactomannan, while SBH contains significant amounts of cellulose, galactomannan, pectin, xylan and xyloglucan[34,35]. The sugar composition of SBH and GG are indicated in Table S2.

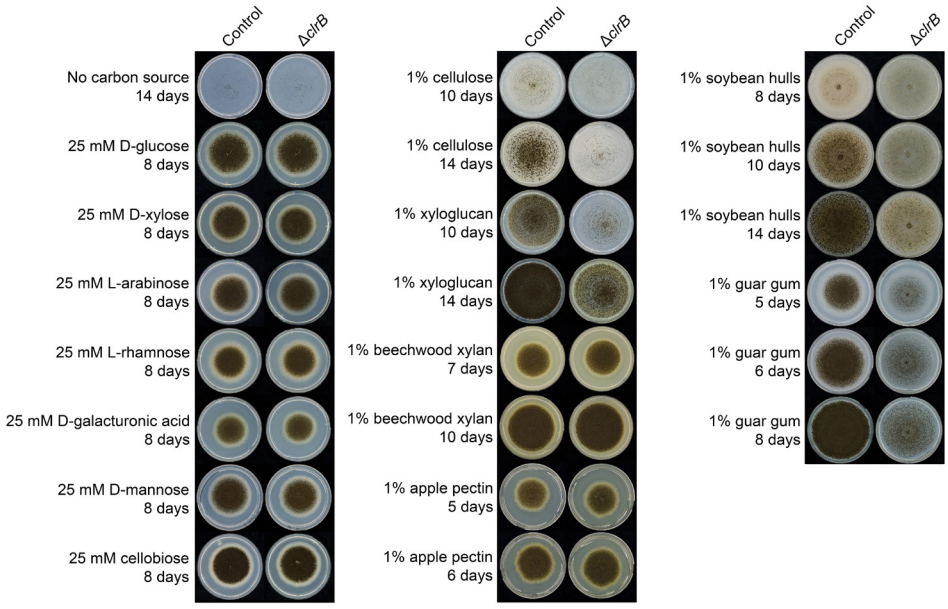


Figure 1. Growth profile of the *A. niger* control (CBS 138852) and Δ*clrB* strain. The solid medium containing selected carbon sources were inoculated with 1000 spores and incubated at 30°C for up to 14 days.

Growth on GG was strongly reduced in the *clrB* deletion mutant (Figure 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*[17,18], which correlates with abolished growth on cellulose in our study (Figure 1). However, no growth reduction was observed on cellulose for an *A. oryzae manR* disruption mutant, despite its involvement in cellulose utilization[36]. 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* (Figure 1). Growth reduction on this substrate was observed in *A. nidulans*[16], but not in *N. crassa*[16], 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 (Figure 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[18], 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 (Figure 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[18]. 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 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[16,19,20,23,36] or mannobiose[20,36]. Mannobiose-mediated activation of ClrB in *A. niger* has been hypothesized before[37], 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 transporter genes indicated no downregulation

(fold change < 0.5; *p*adj < 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[38]. 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[39].

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[40]. Furthermore, the expression of *clrB* is positively affected by the xylose-inducible TF XlnR[17], which could likely explain the role of ClrB in the utilization of diverse substrates containing low levels, or even lacking mannobiose in their composition[17,18,30].

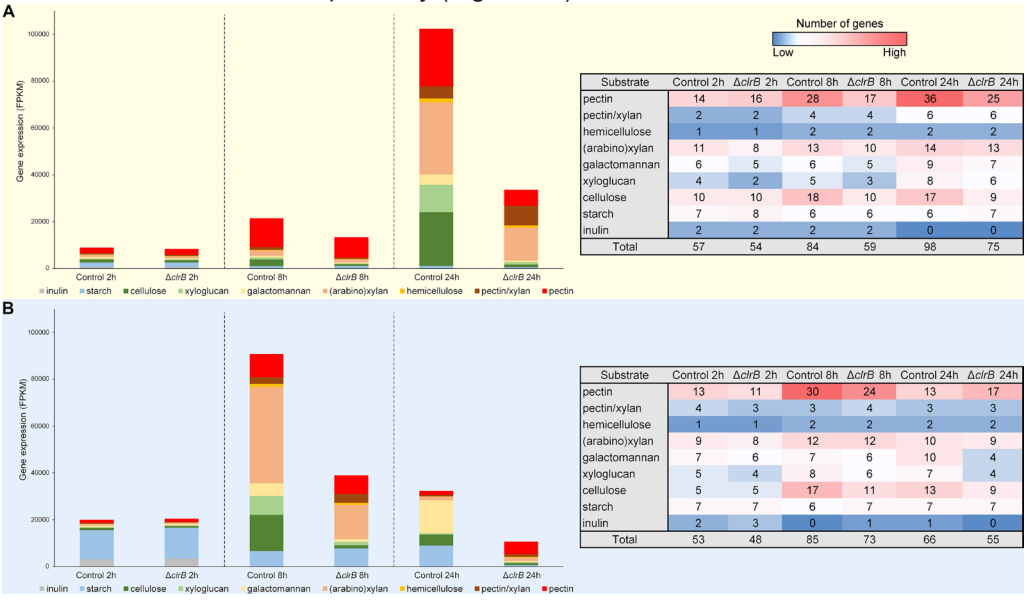
Table 1. Differentially expressed genes in the Δ*clrB* strain compared to the control when cultivated in minimal medium containing 2 mM mannobiose as sole carbon source. Gene expression values represent FPKM values, while the fold change is based on Deseq2 calculation. Enzyme activity abbreviations are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

CAZy-encoding genes								
Gene ID	**Gene name**	**Activity**	**CAZy family**	**Substrate**	**FPKM control**	**FPKM Δ*clrB***	**DeSeq2 fold change**	***p*adj**
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**	***p*adj**
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
Transporter genes								
Gene ID	**Gene name**	**Specificity**			**FPKM control**	**FPKM Δ*clrB***	**DeSeq2 fold change**	***p*adj**
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

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 (Figure 2).

On SBH, the overall expression of CAZyme-encoding genes was low after 2 h, showing an increase after 8 h (Figure 2A). However, at 24 h, a relatively high increase in the expression of CAZy genes was observed, 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[30]. 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 (Figure 2A).



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

Figure 2. Schematic representation of the relative expression of CAZyme-encoding genes in *A. niger* (CBS 138852) and Δ*clrB* strain 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.

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 less than 1% starch in SBH[34,41]. 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 (Figure 2A).

In contrast, after 8 h, the expression of three endo-arabinanase encoding genes (*abnA, abnC* and NRRL3_3855) was the highest (Figure 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 (Figure 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*) (Figure 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 (Figure 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 (Figure 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 (Figure 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*[42]. 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.

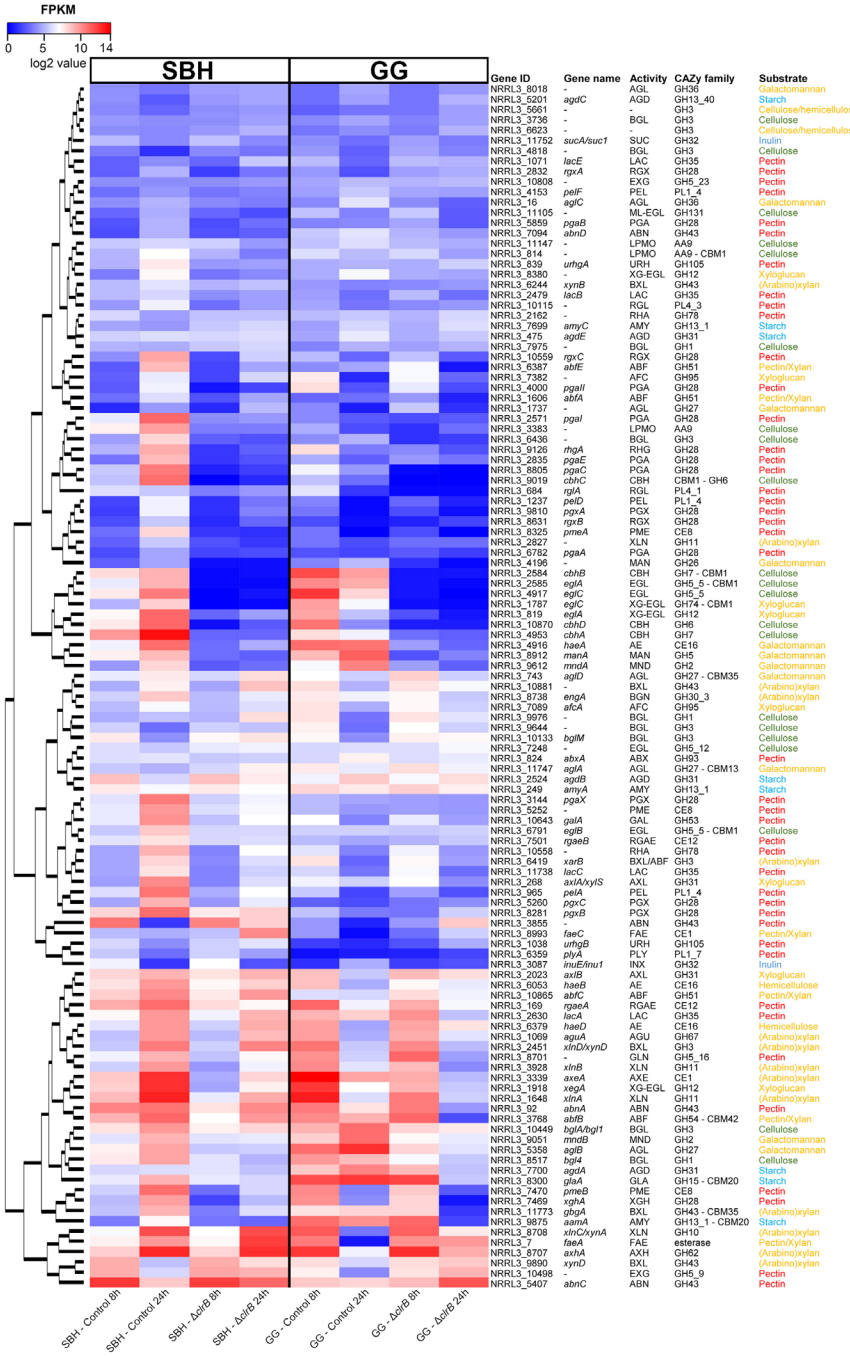


Figure 3. Hierarchical clustering of CAZyme-encoding genes in *A. niger* control (CBS 138852) and Δ*clrB* strain. Data originated from 8 and 24 h of culturing in 1% soybean hulls (SBH) or 1% guar gum (GG) liquid media. The substrates associated with the corresponding genes are indicated by different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

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 (Figure 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* (Figure 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* (Figure 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.

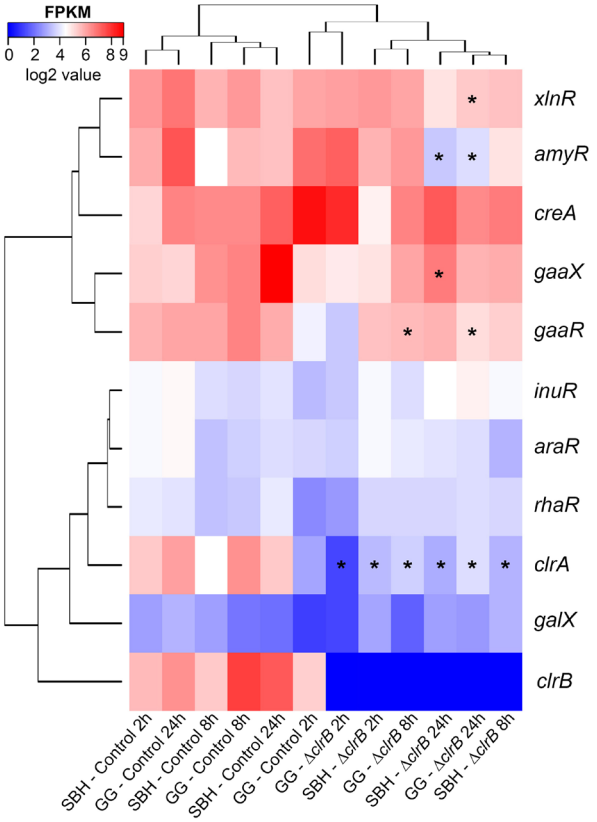


Figure 4. Hierarchical clustering of transcription factor genes in *A. niger* control (CBS 138852) and Δ*clrB* strain. Data originated from 2, 8 and 24 h of culturing in 1% soybean hulls (SBH) or 1% guar gum (GG) liquid media. Downregulated genes (fold change < 0.5; *p*adj < 0.01) in Δ*clrB* compared 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.

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 (Figure 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 (Figure 5 and Table S3).

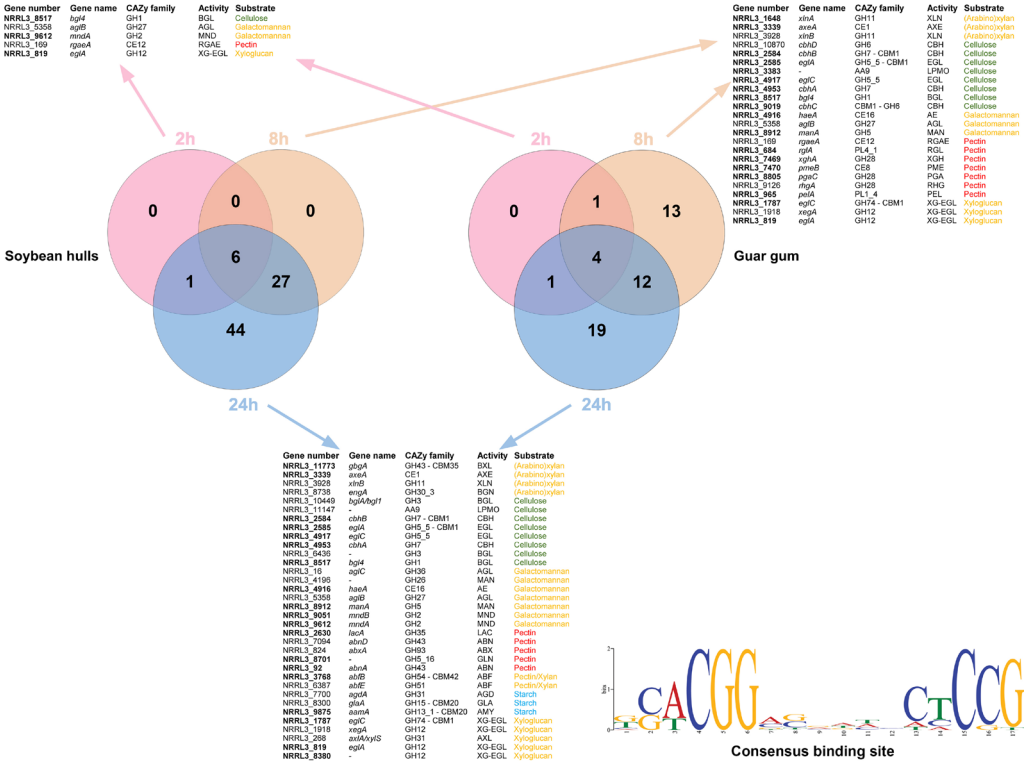


Figure 5. Downregulated CAZyme-encoding genes in *A. niger* grown on 1% soybean hulls or 1% guar gum. Venn-diagrams indicate the number of downregulated (fold change < 0.5; *p*adj < 0.01) genes in Δ*clrB* strain compared to the control at different time points. Only the genes which are downregulated 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.

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 were also downregulated at 24 h, so no unique downregulated genes were found in this mutant at early time points (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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*[17]. However, our study shows that the expression of *clrA* is (partially) dependent on ClrB in *A. niger* (Figure 4). This indicates that the decreased expression of *clrA* in a Δ*xlnR* mutant[17] 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 (Figure 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 (Figure 2A). However, the 24 h time point showed the downregulation of additional galactomannanolytic genes, including *aglC*, NRRL3_4196 (putative endomannanase), *mndA* and *mndB* (Figure 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[40]. Regulation of *manC* and *mndB* by *N. crassa* CLR-2 and *A. nidulans* ClrB has been reported before[19]. 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*[20,36]. 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) (Figure 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[16,19]. However, this was not the case for *A. oryzae* ManR, which showed no significant effect on xylanolytic gene expression on Avicel[36]. 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[21] and *T. cellulolyticus* TclB2[25] 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 (Figure 5). A previous study showed that the expression of *xlnB* (*xynA*) decreased in *A. niger ΔclrB* on wheat straw[17]. 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 (Figure 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[18]. 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[18].

The expression of xyloglucanolytic genes was also affected in Δ*clrB* (Figure 5). Reduced xyloglucanolytic activities are also indicated by the reduced growth of Δ*clrB* on xyloglucan (Figure 1). The importance of CLR-2 in the degradation of xyloglucan was reported before in *N. crassa*[22], 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* (Figure 5). Our predicted consensus binding site [5'-CGGN$_8$CCG-3'] matched the one previously described for *A. nidulans* ClrB[23] and shows high similarity to the experimentally determined [5'-CGGN$_{11}$CCG-3'] binding site in *N. crassa*[43].

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[23], 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 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*[38], 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*[19]. 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).

Table 2. Downregulated 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[44])	Soybean hulls						Guar gum					
			Control 2h	Control 8h	Control 24h	Δ*clrB* 2h	Δ*clrB* 8h	Δ*clrB* 24h	Control 2h	Control 8h	Control 24h	Δ*clrB* 2h	Δ*clrB* 8h	Δ*clrB* 24h
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

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[44]. In contrast, no function has been associated yet with NRRL3_5614 and NRRL3_10866 genes[44]. 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 (Figure 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 coding for CAZymes necessary for the degradation of SBH and GG in *A. niger*. ClrB is essential to maintain growth on cellulose and galacto(mannan) and is highly involved in the degradation of xyloglucan. Gene expression 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*). Furthermore, gene expression data indicate the involvement of ClrB in the regulation of several transporter genes, including *ctA*, but shows no (direct) involvement in the regulation of primary metabolic pathways.

Finally, we show that mannobiose is most likely the inducer of ClrB in *A. niger*. 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 plant biomass degrading enzymes through targeted gene editing and the optimization of culture conditions.

Data availability

The raw RNAseq data were deposited with the Sequence Read Archive at NCBI with sample accession numbers SRP343310-343356. All other data are available in the main text or in the supplementary files.

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.

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Supporting information

All supporting information are available upon request from the author.

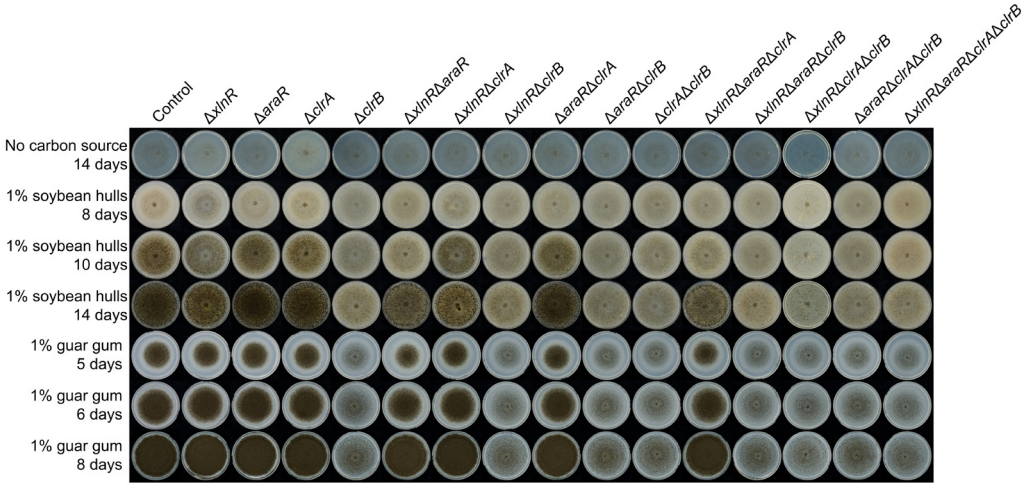


Figure S1. Growth profile of *A. niger* control (CBS 138852) and single and combinatorial deletion mutants on soybean hulls. The solid medium was inoculated with 1000 spores and incubated at 30°C for up to 14 days.

Table S1. *A. niger* strains used in this study.

Table S2. Sugar composition analysis of soybean hulls and guar gum. The analysis was performed as previously described.

Table S3. Downregulated genes in *A. niger* Δ*clrB* strain compared to the control (CBS 138852). Strains were grown for 2, 8 and 24 h in soybean hulls (SBH) and guar gum (GG) liquid cultures. (+) indicates the condition in which the analyzed genes are downregulated (fold change < 0.5; *p*adj < 0.01). The genes which were downregulated on both SBH and GG after 8 and 24 h of growth are highlighted in bold. Genes which were also downregulated on mannobiose are indicated by an asterisk (*).

Gene ID	Gene name	Activity	CAZy family	Substrate	SBH 2h	SBH 8h	SBH 24h	GG 2h	GG 8h	GG 24h	Putative binding sites (CGGN₈CCG)
NRRL3_1069	*aguA*	AGU(α-glucuronidase)	GH67	(Arabino)xylan			+				0
NRRL3_11773*	*gbgA*	BXL(β-1,4-xylosidase)	GH43 - CBM35	(Arabino)xylan		+	+			+	1
NRRL3_1648	*xlnA*	XLN(β-1,4-endo-xylanase)	GH11	(Arabino)xylan		+	+		+		1
NRRL3_2827	-	XLN(β-1,4-endo-xylanase)	GH11	(Arabino)xylan			+				1
NRRL3_3339*	***axeA***	**AXE(acetyl xylan esterase)**	**CE1**	**(Arabino)xylan**	**+**	**+**	**+**		**+**	**+**	**1**
NRRL3_3928	***xlnB***	**XLN(β-1,4-endo-xylanase)**	**GH11**	**(Arabino)xylan**		**+**	**+**		**+**	**+**	**0**
NRRL3_6244	*xynB*	BXL(β-1,4-xylosidase)	GH43	(Arabino)xylan			+				0
NRRL3_6419	*xarB*	BXL/ABF(β-1,4-xylosidase/α-arabinofuranosidase)	GH3	(Arabino)xylan			+				0
NRRL3_8738	*engA*	BGN(endo-1,6-β-D-glucanase)	GH30_3	(Arabino)xylan			+			+	0
NRRL3_10449	*bglA/bgl1*	BGL(β-1,4-glucosidase)	GH3	Cellulose			+		+	+	0
NRRL3_10870*	*cbhD*	CBH(cellobiohydrolase)	GH6	Cellulose		+	+		+		0
NRRL3_11105	-	ML-EGL(β-1,3/β-1,4-endoglucanase)	GH131	Cellulose			+				1
NRRL3_11147	-	LPMO(lytic polysaccharide monooxygenase)	AA9	Cellulose			+			+	0
NRRL3_2584*	***cbhB***	**CBH(cellobiohydrolase)**	**GH7 - CBM1**	**Cellulose**		**+**	**+**		**+**	**+**	**1**
NRRL3_2585*	***eglA***	**EGL(β-1,4-endo-glucanase)**	**GH5_5 - CBM1**	**Cellulose**		**+**	**+**		**+**	**+**	**2**
NRRL3_3383*	-	LPMO(lytic polysaccharide monooxygenase)	AA9	Cellulose		+	+		+		1
NRRL3_4917*	***eglC***	**EGL(β-1,4-endo-glucanase)**	**GH5_5**	**Cellulose**		**+**	**+**		**+**	**+**	**1**
NRRL3_4953	***cbhA***	**CBH(cellobiohydrolase)**	**GH7**	**Cellulose**		**+**	**+**		**+**	**+**	**1**
NRRL3_6436	-	BGL(β-1,4-glucosidase)	GH3	Cellulose		+	+			+	0
NRRL3_6791	*eglB*	EGL(β-1,4-endo-glucanase)	GH5_5 - CBM1	Cellulose			+				0
NRRL3_814	-	LPMO(lytic polysaccharide monooxygenase)	AA9 - CBM1	Cellulose			+				0
NRRL3_8517*	***bgl4***	**BGL(β-1,4-glucosidase)**	**GH1**	**Cellulose**	**+**	**+**	**+**	**+**	**+**	**+**	**1**
NRRL3_9019	*cbhC*	CBH(cellobiohydrolase)	CBM1 - GH6	Cellulose		+	+		+		3
NRRL3_16*	*aglC*	AGL(α-1,4-galactosidase)	GH36	Galactomannan			+			+	0
NRRL3_4196	-	MAN(β-1,4-endo-mannanase)	GH26	Galactomannan			+	+		+	0
NRRL3_5358*	***aglB***	**AGL(α-1,4-galactosidase)**	**GH27**	**Galactomannan**	**+**	**+**	**+**	**+**	**+**	**+**	**0**
NRRL3_8912*	***manA***	**MAN(β-1,4-endo-mannanase)**	**GH5**	**Galactomannan**		**+**	**+**		**+**	**+**	**1**
NRRL3_9051*	*mndB*	MND(β-1,4-mannosidase)	GH2	Galactomannan			+		+	+	2
NRRL3_9612*	*mndA*	MND(β-1,4-mannosidase)	GH2	Galactomannan	+		+	+	+	+	1
NRRL3_4916*	***haeA***	**AE (acetyl esterase)**	**CE16**	**Galactomannan**		**+**	**+**		**+**	**+**	**1**
NRRL3_6053	*haeB*	AE (acetyl esterase)	CE16	Hemicellulose			+		+		1
NRRL3_3087	*inuE*	INX(exo-inulinase)	GH32	Inulin						+	0
NRRL3_10115	-	RGL(rhamnogalacturonan lyase)	PL4_3	Pectin			+				3
NRRL3_10558	-	RHA(α-rhamnosidase)	GH78	Pectin		+	+				0
NRRL3_10559	*rgxC*	RGX(exo-rhamnogalacturonase)	GH28	Pectin			+				0
NRRL3_10643	*galA*	GAL(β-1,4-endo-galactanase)	GH53	Pectin		+	+				1
NRRL3_11738	*lacC*	LAC(β-1,4-galactosidase)	GH35	Pectin			+				1
NRRL3_1237	*pelD*	PEL(pectin lyase)	PL1_4	Pectin			+				1
NRRL3_169*	*rgaeA*	RGAE(rhamnogalacturonan acetyl esterase)	CE12	Pectin	+	+	+	+	+		0
NRRL3_2479	*lacB*	LAC(β-1,4-galactosidase)	GH35	Pectin		+	+				0
NRRL3_2571	*pgaI*	PGA(endo-polygalacturonase)	GH28	Pectin		+	+				0
NRRL3_2630	*lacA*	LAC(β-1,4-galactosidase)	GH35	Pectin			+			+	1
NRRL3_2832	*rgxA*	RGX(exo-rhamnogalacturonase)	GH28	Pectin			+				0
NRRL3_2835	*pgaE*	PGA(endo-polygalacturonase)	GH28	Pectin			+				1
NRRL3_3144	*pgaX*	PGX(exo-polygalacturonase)	GH28	Pectin			+				0
NRRL3_4000	*pgaII*	PGA(endo-polygalacturonase)	GH28	Pectin			+		+		0
NRRL3_5252	-	PME(pectin methyl esterase)	CE8	Pectin			+				0
NRRL3_5260	*pgxC*	PGX(exo-polygalacturonase)	GH28	Pectin			+				0
NRRL3_5859	*pgaB*	PGA(endo-polygalacturonase)	GH28	Pectin			+				0
NRRL3_6782	*pgaA*	PGA(endo-polygalacturonase)	GH28	Pectin			+				0
NRRL3_684*	*rglA*	RGL(rhamnogalacturonan lyase)	PL4_1	Pectin		+	+		+		1
NRRL3_7094	*abnD*	ABN(endo-arabinanase)	GH43	Pectin			+			+	0
NRRL3_7469	*xghA*	XGH(xylogalacturonase)	GH28	Pectin		+	+		+		1
NRRL3_7470	*pmeB*	PME(pectin methyl esterase)	CE8	Pectin		+	+		+		1
NRRL3_7501	*rgaeB*	RGAE(rhamnogalacturonan acetyl esterase)	CE12	Pectin			+				0
NRRL3_824	*abxA*	ABX	GH93	Pectin			+			+	0
NRRL3_8281	*pgxB*	PGX(exo-polygalacturonase)	GH28	Pectin			+		+		0
NRRL3_8325	*pmeA*	PME(pectin methyl esterase)	CE8	Pectin			+				0
NRRL3_839	*urhgA*	URH(unsaturated rhamnogalacturonyl hydrolase)	GH105	Pectin			+				0
NRRL3_8631	*rgxB*	RGX(exo-rhamnogalacturonase)	GH28	Pectin			+				0
NRRL3_8701	-	GLN(exo 1,6 galactanase)	GH5_16	Pectin		+	+			+	1
NRRL3_8805	*pgaC*	PGA(endo-polygalacturonase)	GH28	Pectin		+	+		+		1
NRRL3_9126	*rhgA*	RHG(endo-rhamnogalacturonase)	GH28	Pectin		+	+		+		0
NRRL3_92*	*abnA*	ABN(endo-arabinanase)	GH43	Pectin	+	+	+			+	1
NRRL3_965	*pelA*	PEL(pectin lyase)	PL1_4	Pectin		+	+		+		1
NRRL3_9810	*pgxA*	PGX(exo-polygalacturonase)	GH28	Pectin			+				0
NRRL3_4153	*pelF*	PEL(pectin lyase)	PL1_4	Pectin						+	0
NRRL3_1606	*abfA*	ABF(α-arabinofuranosidase)	GH51	Pectin/Xylan			+				0
NRRL3_3768	*abfB*	ABF(α-arabinofuranosidase)	GH54 - CBM42	Pectin/Xylan		+	+			+	2
NRRL3_6387	*abfE*	ABF(α-arabinofuranosidase)	GH51	Pectin/Xylan			+			+	0
NRRL3_7700	*agdA*	AGD(α-glucosidase)	GH31	Starch			+			+	0
NRRL3_8300	*glaA*	GLA(glucoamylase)	GH15 - CBM20	Starch			+			+	0
NRRL3_9875	*aamA*	AMY(α-amylase)	GH13_1 - CBM20	Starch			+			+	1
NRRL3_1787	***eglC***	**XG-EGL(xyloglucanase)**	**GH74 - CBM1**	**Xyloglucan**		**+**	**+**		**+**	**+**	**2**
NRRL3_1918*	***xegA***	**XG-EGL(xyloglucanase)**	**GH12**	**Xyloglucan**		**+**	**+**		**+**	**+**	**0**
NRRL3_268	*axlA/xylS*	AXL(α-xylosidase)	GH31	Xyloglucan			+			+	0
NRRL3_7089	*afcA*	AFC(α-L-fucosidase)	GH95	Xyloglucan			+				0
NRRL3_7382	-	AFC(α-L-fucosidase)	GH95	Xyloglucan			+				0
NRRL3_819	***eglA***	**XG-EGL(xyloglucanase)**	**GH12**	**Xyloglucan**	**+**	**+**	**+**	**+**	**+**	**+**	**1**
NRRL3_8380	-	XG-EGL(xyloglucanase)	GH12	Xyloglucan			+			+	1

Table S4. Abbreviations of enzyme activities presented in this study.

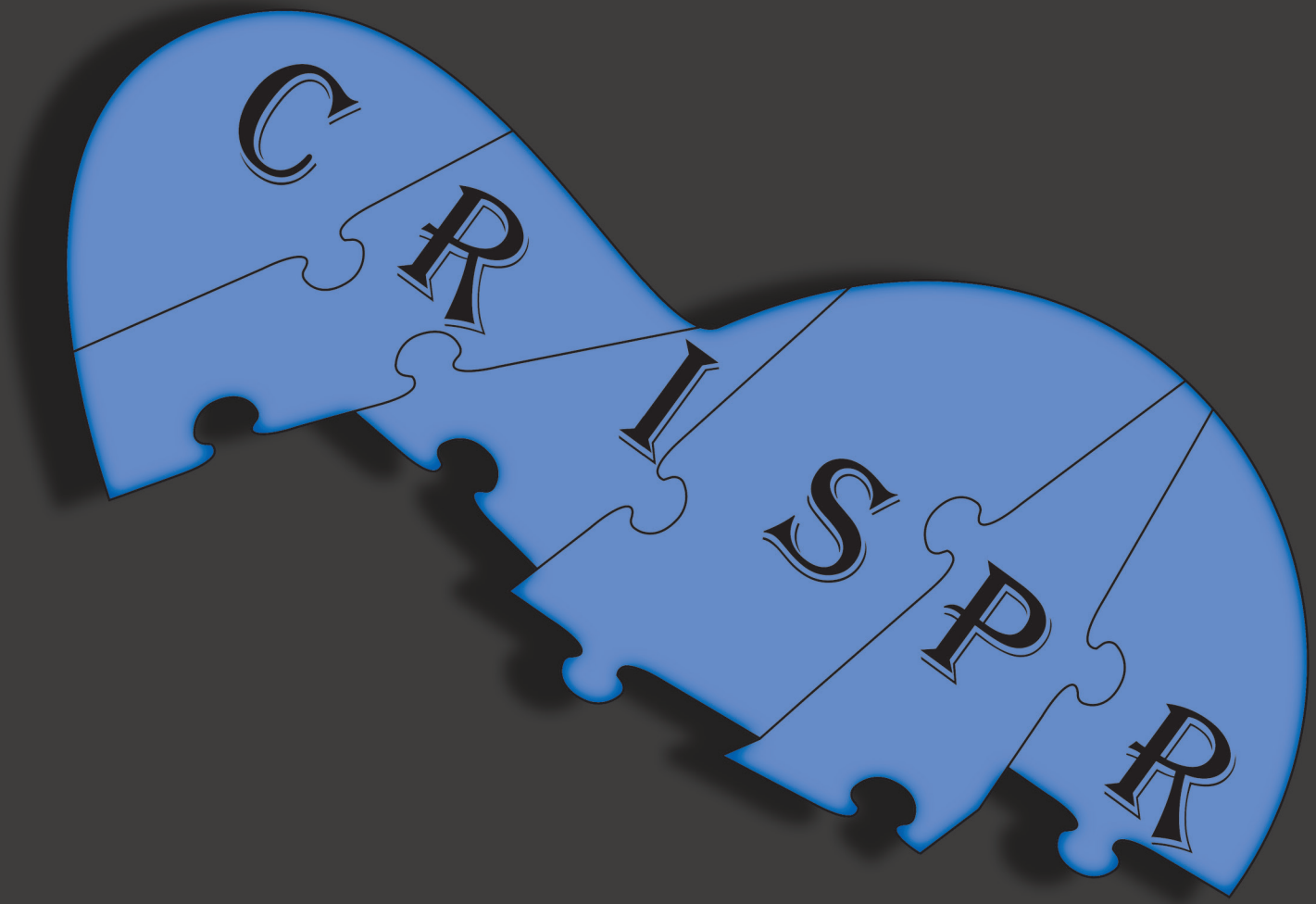
Data S1A. Gene expression analysis of *A. niger* control (CBS 138852) and Δ*clrB* cultivated on 2 mM cellobiose (CB) or 2 mM mannobiose (MB) for 1 h. Transporter, metabolic, CAZy and transcription factor-encoding genes are indicated in different colors. Genes with an expression value of FPKM < 20 were considered low and are indicated in red. Upregulated genes (fold change > 2) are indicated in blue, while downregulated genes (fold change < 0.5) are indicated in orange. Statistically significant changes (*p*adj < 0.01) are indicated in green.

Data S1B. Expression analysis of CAZy-encoding genes in *A. niger* control (CBS 138852) and Δ*clrB* strain. Samples originate from 1% soybean hulls (SBH) or 1% guar gum (GG) cultures, incubated for 2, 8 or 24 h. Genes with an expression value of FPKM < 20 were considered low and are indicated in red. Upregulated genes (fold change > 2) are indicated in blue, while downregulated genes (fold change < 0.5) are indicated in orange. Statistically significant changes (*p*adj < 0.01) are indicated in green.

Data S1C. Expression analysis of transcription factor genes in *A. niger* control (CBS 138852) and Δ*clrB* strain. Samples originate from 1% soybean hulls (SBH) or 1% guar gum (GG) cultures, incubated for 2, 8 or 24 h. Genes with an expression value of FPKM < 20 were considered low and are indicated in red. Downregulated genes (fold change < 0.5) are indicated in orange. Statistically significant changes (*p*adj < 0.01) are indicated in green.

Data S1D. Expression analysis of transporter genes in *A. niger* control (CBS 138852) and Δ*clrB* strain. Samples originate from 1% soybean hulls (SBH) or 1% guar gum (GG) cultures, incubated for 2, 8 or 24 h. Genes with an expression value of FPKM < 20 were considered low and are indicated in red. Upregulated genes (fold change > 2) are indicated in blue, while downregulated genes (fold change < 0.5) are indicated in orange. Statistically significant changes (*p*adj < 0.01) are indicated in green.

Data S1E. Expression analysis of metabolic genes in *A. niger* control (CBS 138852) and Δ*clrB* strain. Samples originate from 1% soybean hulls (SBH) or 1% guar gum (GG) cultures, incubated for 2, 8 or 24 h. Genes with an expression value of FPKM < 20 were considered low and are indicated in red. Upregulated genes (fold change > 2) are indicated in blue, while downregulated genes (fold change < 0.5) are indicated in orange. Statistically significant changes (*p*adj < 0.01) are indicated in green.


CRISPR

Chapter 6

The amylolytic regulator AmyR of *Aspergillus niger* is involved in sucrose and inulin utilization in a culture condition-dependent manner

Submitted to *Fungal Genetics and Biology*

Roland S. Kun, Sonia Salazar-Cerezo, Mao Peng, Yu Zhang, Emily Savage, Anna Lipzen, Vivian Ng, Igor V. Grigoriev, Ronald P. de Vries and Sandra Garrigues

Abstract

Filamentous fungi degrade complex plant material to its monomeric building blocks, which have many biotechnological applications. Transcription factors (TFs) play a key role in plant biomass degradation in fungi, and several TFs have been reported to control the degradation of polysaccharides such as xylan, cellulose, pectin or starch. However, little is known about the interaction of TFs in the regulation of polysaccharide degradation. Here, we studied the regulators of storage-polysaccharide utilization, AmyR and InuR in *Aspergillus niger*. So far, independent regulatory functions have been assigned to these two TFs. AmyR has been described to control starch degradation, while InuR was identified as a regulator involved in the utilization of sucrose and inulin. In our study, the phenotypes of the parental *A. niger*, and Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* mutants were evaluated on media containing sucrose or inulin as carbon source to evaluate the roles of AmyR and InuR in substrate utilization. Moreover, each strain was grown on both solid and liquid media to assess the effect of culture conditions on their roles. In correlation with previous studies, our data shows that AmyR has a minor contribution to sucrose and inulin utilization when InuR is active. In contrast, growth evaluation and transcriptomic data showed that the deletion of *amyR* in the Δ*inuR* background strain resulted in further reduced growth ability on both substrates, mainly evidenced by data originated from solid plate cultures.

1. Introduction

Plant biomass is the most abundant carbon source on Earth and is mainly composed of polysaccharides (cellulose, hemicellulose and pectin), the aromatic compound lignin, and storage polymers, such as starch and inulin[1,2]. Filamentous fungi secrete a wide arsenal of hydrolytic and oxidative enzymes that degrade these complex plant materials, and many of these enzymes have been commercially used for several industrial and biotechnological applications, such as pulp and paper, food and feed, detergents, textile and biofuel and biochemicals[3,4].

Starch degradation is performed by the production of amylolytic enzymes, (e.g., α-amylases, glucoamylases, and α-glucosidases) which are mainly classified into the families of glycoside hydrolases GH13, GH15 and GH31 (www.cazy.org)[5,6]. Starch degradation is regulated at the transcriptional level in *Aspergillus* mainly by the transcriptional activator AmyR[7,8], which was the first GAL4-like transcription factor (TF) identified in filamentous fungi[9]. The role of AmyR in starch degradation has been studied in the last decades in many *Aspergillus* species[10–12], although a broader role of this regulator was observed in *Aspergillus niger*[8,13–15]. AmyR triggers the production of amylolytic enzymes due to the presence of starch, maltose or low levels of D-glucose, which act as inducer compounds[8,16,17].

Inulin is another reserve carbohydrate found in plants, especially chicory, dahlia, and Jerusalem artichoke[18]. Inulin-acting enzymes, such as exo-inulinases, inulin lyases, and invertases are fructofuranosyl hydrolases that target the β-2,1 linkages of inulin and hydrolyze it into fructose and glucose[19], and are an important class of industrial enzymes belonging to family GH32. The production of these enzymes is induced in the presence of inulin or sucrose, and has been reported to be under the control of a complex regulatory system[20,21], where the TF InuR plays the most dominant role. InuR acts as a positive transcriptional activator for the expression of genes involved in the breakdown of inulin and sucrose and the uptake of inulin-derived compounds, and has been reported to be closely related to AmyR[22]. These two regulators share structural similarities and putative DNA binding sites ($CGGN_8[C/A]GG$)[12,22,23], indicating that they could have originated form the same ancestor gene. Despite these similarities, gene co-regulation or possible interaction of AmyR and InuR in the regulation of starch or inulin degradation has not been assessed yet in filamentous fungi.

In this study, we evaluated the influence of the amylolytic TF AmyR on the utilization of sucrose and inulin in the industrially relevant fungus *A. niger*. For this, single Δ*amyR* and Δ*inuR* deletion mutants, and the double Δ*amyR*Δ*inuR* deletion mutant were

obtained using CRISPR/Cas9 genome editing technology[24], and phenotypical and transcriptomic analyses were performed. It has been previously shown that different cultivation methods involving the use of solid or liquid medium can highly affect the expression of genes involved in substrate degradation[25]. Therefore, we assessed the genetic response of the analyzed strains to growth on both solid or liquid medium containing sucrose or inulin as carbon source. Results showed that AmyR contributes to the utilization of sucrose and inulin, which is represented by the reduced growth and expression levels of Carbohydrate Active enZyme (CAZyme)-encoding genes when the fungus was grown on solid substrates.

2. Materials and methods

2.1 Strains, media and growth conditions

Escherichia coli DH5α was grown in Luria-Bertani (LB) medium supplemented with 50 µg/mL ampicillin (Sigma-Aldrich), and was used for plasmid propagation. *Aspergillus niger* CBS 138852[26] was used as a parental strain for construction of the mutants. The generated *A. niger* Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* mutants were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute and the accession numbers are shown in Table S1.

For strain propagation, *A. niger* control (CBS 138852), Δ*amyR*, Δ*inuR,* and Δ*amyR*Δ*inuR* strains were cultured in *Aspergillus* Minimal Medium (MM) or Complete Medium (CM)[27] at 30°C supplemented with 1% D-glucose and 1.22 g/L uridine (Sigma-Aldrich). Conidia were harvested and dispersed in ACES buffer, and their concentration was adjusted using a haemocytometer.

Growth profiles were carried out using MM containing 25 mM D-glucose, D-fructose, sucrose or maltose (Sigma-Aldrich), and 1% soluble starch (Difco™) or inulin from chicory (Sigma-Aldrich). All media were supplemented with 1.22 g/L uridine. Plates were inoculated in duplicates with 2 µL containing 10^3 conidia and incubated at 30ºC for up to 8 days. Plates were evaluated daily by visual inspection, taking into account colony diameter, mycelial density and sporulation. Pictures were taken after 8 days of incubation.

2.2 DNA construction and fungal transformation

The ANEp8-Cas9-*pyrG* plasmid, which contains the autonomous fungal replicating element AMA1, *pyrG* as selection marker, *cas9* gene, and the single guide RNA (sgRNA) expression construct under the control of the proline transfer ribonucleic acid ($tRNA^{Pro1}$) promoter was used in this study for the generation of fungal transformants[24]. The design of the 20 bp protospacers for the sgRNAs were performed using the Geneious 11.04.4 software tool (https://www.geneious.com). The sgRNA sequences (Table S2) with no predicted off-targets and the highest on-target activity were designed based on the experimentally determined predictive model described by Doench et al.[28]. The sgRNAs for each gene were obtained to delete *amyR* (gene ID: NRRL3_07701) and *inuR* (gene ID: NRRL3_03593) genes in *A. niger* CBS 138852. All repair templates (RTs), which include the 5' and 3' flanking regions of the target genes, were obtained by fusion-PCR using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Two PCR fragments were generated by amplifying approx. 750 bp upstream and downstream of the *inuR* and *amyR* genes. These two fragments were fused together in a second nested PCR obtaining ~1,200 bp RT, and were subsequently purified (Wizard® SV Gel and PCR Clean-Up System, Promega).

CRISPR/Cas9 plasmid construction, generation of *A. niger* protoplasts, transformation and purification of putative mutant strains were performed as previously described[29] with minor modifications[30]. Mutant strains were confirmed by PCR through the amplification of target gene region (Figure 1). All primers used in this study are shown in Table S2 and were ordered from Integrated DNA Technologies (IDT, Leuven, Belgium).

2.3 Mycelial dry weight measurement

For mycelial dry weight measurements from liquid cultures, 10^6 conidia/mL of *A. niger* control, Δ*amyR*, Δ*inuR*, and Δ*amyR*Δ*inuR* strains were inoculated in 50 mL MM containing 1% sucrose or 1% inulin supplemented with 1.22 g/L uridine and 5 mM D-fructose to facilitate fungal germination. Triplicate samples were harvested after 48 h of cultivation in a rotary shaker at 250 rpm and 30°C. For mycelial dry weight measurements from solid cultures, 10^3 conidia of *A. niger* control, Δ*amyR*, Δ*inuR*, and Δ*amyR*Δ*inuR* strains were inoculated between two sterile Polycarbonate Track Etched (PCTE) membrane layers (disc diameter 76 mm, PCTE 0.1 µm, Poretics™, GVS Filter Technology) on MM plates containing 1% inulin or 1% sucrose as carbon source supplemented with 1.22 g/L uridine and 5 mM D-fructose. Non-sporulated mycelia were

collected after 7 days of growth at 30°C. Plates were inoculated in technical duplicates. Mycelia obtained from both liquid and solid cultures were subsequently dried o/n at 60ºC and weighed. Statistical significance was determined using Student's two-tailed type II *t*-test. Significance was regarded as $p < 0.05$.

2.4 SDS-PAGE assays

For protein production analysis, 10^6 spores/mL of *A. niger* control, Δ*amyR*, Δ*inuR,* and Δ*amyR*Δ*inuR* mutant strains were pre-cultured in 250 mL CM containing 2% D-xylose and 1.22 g/L uridine for 16 h at 30°C and 250 rpm. Mycelia (~2.5 g wet weight) were transferred to 50 mL MM containing 1% inulin or 1% soluble starch as carbon source, supplemented with 1.22 g/L uridine, and were incubated at 30°C and 250 rpm. Supernatant samples were taken after 4, 8, 24 and 32 h of incubation and were centrifuged for 10 min at 13,500 × *g*. Ten µL of each sample were analyzed by SDS-PAGE using SDS-12% polyacrylamide gels calibrated with PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) and silver stained[31]. Samples were evaluated in biological triplicates.

2.5 Transcriptomics analysis

For transcriptomics analysis of liquid culture samples, freshly harvested spores from *A. niger* control, Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains were pre-grown in 250 mL CM containing 2% D-xylose and 1.22 g/L uridine for 16 h at 30°C in a rotary shaker at 250 rpm. After 16 hours, mycelia were harvested by filtration through sterile cheesecloth, rinsed with MM, and approximately 2.5 g (wet weight) mycelium was transferred into 50 mL MM containing 1% sucrose, and 1% inulin. Mycelia were collected after 2 and 8 h and were frozen in liquid nitrogen followed by storage at -80°C. Samples were collected in triplicates.

For transcriptomics analysis of solid culture samples, 10^3 conidia of *A. niger* control, Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains were inoculated and pre-grown between two sterile Polycarbonate Track Etched (PCTE) membrane layers (disc diameter 76 mm, PCTE 0.1 µm, Poretics™, GVS Filter Technology) on MM plates containing 1% soybean hulls as carbon source supplemented with 1.22 g/L uridine at 30°C. After two days, the polycarbonate membrane layers containing the fungal mycelia were transferred to MM plates containing 1% sucrose or 1% inulin supplemented with 1.22 g/L uridine and were grown at 30°C. Mycelia were collected after 40 h of growth and were frozen in liquid

nitrogen and stored at -80°C. Samples were collected in triplicates.

The transcriptomes of the control and deletion mutant strains cultivated for 2 and 8 h in liquid cultures and cultivated for 40 h on solid cultures were analyzed using RNA-seq. RNA isolation, purification and quantitative and qualitative evaluation was performed as previously described[32].

For liquid culture samples, purification of mRNA isolated from liquid cultures, synthesis of cDNA library and sequencing were performed at the Joint Genome Institute (JGI, US). RNA sequencing, processing of raw fastq reads and evaluation of raw gene counts were performed as previously reported[32]. Three biological replicates were generated and sequenced for each condition. Two individual samples were discarded from further analysis due to their poor sequencing quality.

For solid culture samples, the preparation of libraries and sequencing of the mRNA along with the analysis of the raw data was performed by GenomeScan B.V. The RNA concentration and integrity was assessed using the Agilent Fragment Analyzer system. Subsequently, samples were processed using the NEBNext Ultra II directional RNA library prep kit for Illumina®. Briefly, rRNA was depleted from total RNA using the rRNA depletion kit (Qiagen fast select). After fragmentation of the rRNA reduced RNA, a cDNA synthesis was performed. This was used for ligation with the sequencing adapters and PCR amplification of the resulting product. The quality and yield after sample preparation was measured with the Fragment Analyzer. The size of the resulting products was consistent with the expected size distribution (a broad peak between 300-500 bp). Clustering and DNA sequencing were performed using Illumina Novaseq6000 in line with manufacturer's instructions at the concentration of 1.1nM of DNA. Image analysis, base calling and the quality check were conducted using the Illumina data analysis pipeline RTAv3.4.4 and Bclfastqv2.20. Data obtained from the Novaseq6000 in fastq format was used as source for the downstream data analysis. Alignment of fastq reads was performed using STAR2 version v2.5.4b against the *A. niger* NRRL3. The aligned reads are stored in a sorted BAM format and indexed using Samtools v1.95. For the removal of PCR duplicates within the library Picard MarkDuplicates v2.23.66 was used. This tool uses UMI sequences (BAM tags) to determine which sequences are PCR duplicates and therefore should be removed. The deduplicated mapped reads are counted for each exonic feature in the reference GTF annotation using htseq-count v0.11.07.

In all cases, differentially expressed genes (DEGs) were detected using the R package DESeq2[33]. Transcripts were considered differentially expressed if the DESeq2 fold

change of the deletion mutant strains 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.

3. Results and discussion

3.1 Generation of Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains in *A. niger* in a single transformation event

In order to obtain *A. niger* Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains, one single transformation event was performed in which 1 µg of each ANEp8-Cas9-*pyrG* plasmid carrying sgRNAs targeting *amyR* or *inuR* were mixed and co-transformed together with 5 µg of each corresponding RT into *A. niger* protoplasts as previously described[30]. Forty-eight transformants were obtained and ten were further isolated to monosporic culture and screened (data not shown). From these ten, six transformant colonies were positive Δ*amyR* mutants, two were positive Δ*inuR* mutants and two were double Δ*amyR*Δ*inuR* mutants (Figure 1). All single and combinatorial mutants were identified by PCR using specific primers designed to differentiate between mutants and the control strain (Table S2) (Figure 1).

3.2 The amylolytic transcription factor AmyR influences growth of *A. niger* on sucrose and inulin

To evaluate the contribution of the amylolytic transcriptional activator AmyR and the inulinolytic transcriptional activator InuR to the utilization of storage polysaccharides and related carbon sources in *A. niger*, the parental, Δ*amyR*, Δ*inuR* and the double Δ*amyR*Δ*inuR* strains were grown on agar plates containing different carbon sources. These include D-glucose, D-fructose, sucrose, maltose, starch and inulin (Figure 2). Growth on D-glucose and D-fructose was similar for all strains, whereas Δ*inuR* showed reduced growth on sucrose and inulin compared to the control and Δ*amyR* strains, as previously reported[22].

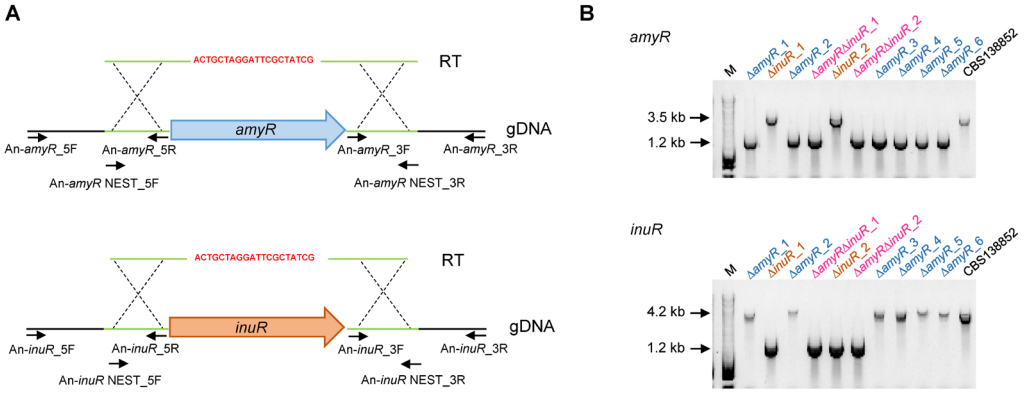


Figure 1. Molecular characterization of *A. niger* Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains. A) Schematic representation of the genomic DNA (gDNA) reparation by homologous recombination after Cas9 cleavage in the *amyR* (blue arrow) and *inuR* (orange arrow) genes. The ~600 bp complementary arms to the upstream and downstream regions of *amyR* and *inuR* are represented in green. To generate the repair template (RT), a 20-bp sequence (red) was introduced for complementarity during fusion PCR. Black arrows represent the primers used for RT generation and transformant confirmation by PCR (Table S2). Figure is not drawn to scale. **B**) PCR confirmation of the *A. niger* Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains. Low bands (~1.2 kb) correspond to the deletants in *amyR* (top panel) and *inuR* (bottom panel), while upper bands (~ 3.5 and 4.2 kb) correspond to the parental strain (CBS 138852). Primer pairs used are located as indicated in the figure.

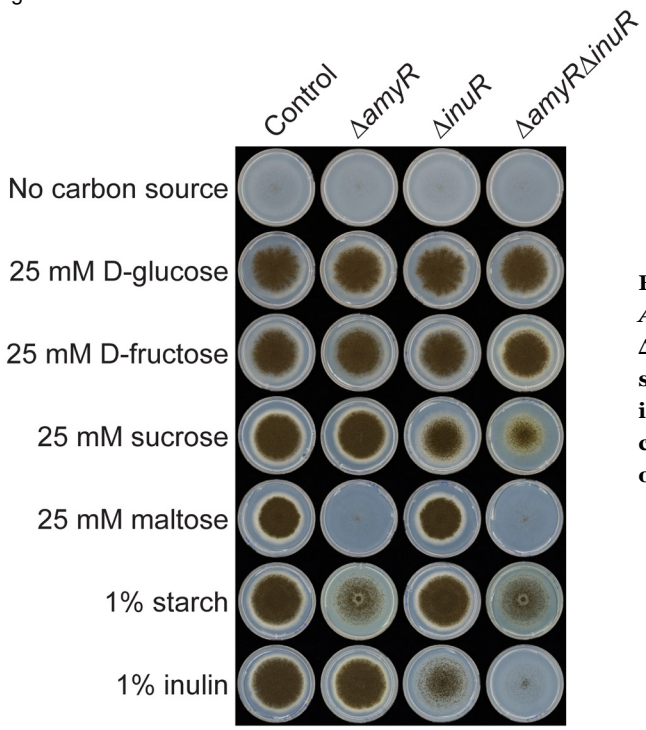


Figure 2. Growth profile of *A. niger* control, Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains grown on starch, inulin and other related carbon sources after 8 days of incubation at 30°C.

These results confirm the involvement of InuR in sucrose and inulin utilization in *A. niger*. The Δ*amyR*Δ*inuR* double deletion strain showed a further reduced growth on both sucrose and inulin compared to the single Δ*inuR* strain, suggesting a role for AmyR in sucrose and inulin utilization in *A. niger*. Finally, the growth on maltose and starch was only affected by the deletion of *amyR*, confirming the dominant role of AmyR in starch utilization[10,11].

3.3 The contribution of AmyR to sucrose and inulin utilization depends on the cultivation method

In order to evaluate the influence of AmyR on growth on sucrose and inulin, the mycelial weight of *A. niger* control, Δ*amyR*, Δ*inuR* and the double Δ*amyR*Δ*inuR* strains was evaluated from both liquid and solid medium cultures (Figure 3). In both cases, the media contained either 1% sucrose or 1% inulin as carbon source, supplemented with 5 mM D-fructose to facilitate initial germination of fungal spores.

In case of liquid cultures, the deletion of *inuR* resulted in substantially decreased mycelial weight in liquid minimal medium (MM) containing 1% sucrose or inulin (average weight of 32 mg and 15 mg, respectively) compared to the control (117 mg and 107 mg, respectively) after 48 h of growth ($p < 0.05$) (Figure 3A). In contrast, dry weight measurement results from liquid cultures did not suggest the contribution of AmyR to growth on sucrose or inulin, indicated by the comparable growth of Δ*amyR* and Δ*amyR*Δ*inuR* strains to that of the control and Δ*inuR* strain, respectively (Figure 3A). SDS-PAGE analysis showed reduced protein production by the Δ*amyR*Δ*inuR* mutant compared to the Δ*inuR* strain after 24 h and 32 h of growth (Figure S1). However, the mycelial weight measurement results indicate that most proteins abolished by the additional deletion of *amyR* in the Δ*inuR* strain are most likely not essential for inulin utilization.

In contrast to the dry weight measurements from liquid cultures, when the tested strains were cultivated on solid MM plates containing the same carbon sources, the Δ*amyR* strain showed reduced mycelial weight on both substrates compared to the control (Figure 3B). Moreover, the Δ*amyR*Δ*inuR* double deletion strain showed significantly reduced mycelial weight on sucrose compared to the Δ*inuR* deletion strain (2.5 mg compared to 27 mg) ($p < 0.05$), which correlates with our growth profile results (Figure 2). Despite the reduced mycelial weight of the Δ*amyR* strain compared to the control on inulin (Figure 3B) and the reduced growth of Δ*amyR*Δ*inuR* mutant compared to

the Δ*inuR* strain on the same substrate (Figure 2), the mycelial weight of Δ*inuR* and Δ*amyR*Δ*inuR* was comparable on inulin in both culture conditions. Overall, these results indicate that AmyR can highly influence the growth on sucrose, and to a lesser extent on inulin, but only when the fungus is cultivated on solid medium.

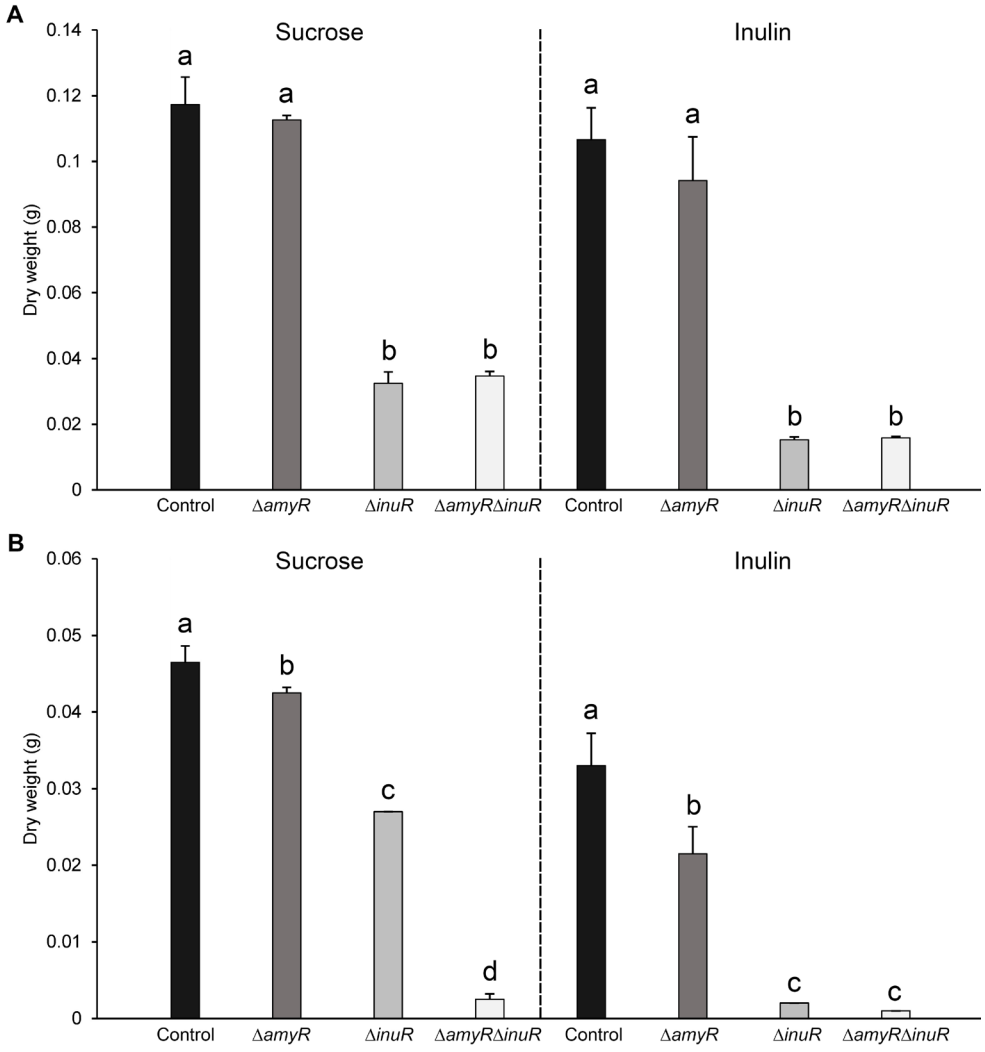


Figure 3. Mycelial dry weight measurement results of *A. niger* control (CBS 138852), Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains grown on liquid (A) or solid (B) medium containing 1% sucrose or inulin as carbon source, supplemented with 1.22 g/L uridine and 5 mM D-fructose to facilitate initial germination of fungal conidia. Liquid culture triplicates were incubated for 48 h in a rotary shaker at 250 rpm and 30°C, while solid medium cultures were grown for 7 days at 30 ºC. Samples showing different letters (a-d) show statistically significant differences among the strains (Student's two-tailed type II *t*-test, $p < 0.05$).

3.4 *A. niger* AmyR shows minor involvement in the regulation of gene expression when the fungus is cultivated in liquid medium containing sucrose and inulin

In order to evaluate the regulatory mechanisms playing a role in sucrose and inulin utilization on a molecular level, transcriptome data were generated from liquid cultures of *A. niger* control, Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains. Samples were taken after 2 and 8 h of growth following a transfer from CM medium containing 2% D-xylose to MM medium containing 1% sucrose or 1% inulin.

Considering the genome wide response of the fungus, the deletion of either *amyR*, *inuR* or both resulted in relatively low number of DEGs compared to the control on inulin after 2 h of growth (Figure 4A, right panel). However, after 8 h of growth, the double deletion of *amyR* and *inuR* resulted in the upregulation of 1995 and downregulation of 1955 genes, which can mostly be associated with the deletion of *inuR*, showing the upregulation and downregulation of 1575 and 1708 genes, respectively (Figure 4A, right panel). In contrast to the inulin culture, the number of DEGs was substantially higher at both timepoints when grown on medium containing 1% sucrose as carbon source, mainly affected by the deletion of *inuR* (Figure 4A, left panel). Interestingly, the Δ*inuR* strain showed a higher number of downregulated genes compared to the Δ*amyR*Δ*inuR* strain after 8 h of cultivation on sucrose, highlighting the major role of InuR for the utilization of this substrate (Figure 4A, left panel). The large number of upregulated genes in the *inuR* single and double deletion mutants correlates with the upregulation of major regulators involved in plant biomass degradation (Figure S2). The upregulation of major TF genes might be an indirect consequence of the downregulation of *creA*, resulting in a carbon catabolite de-repressed phenotype[34] (Figure S2). The relation of AmyR and InuR to the utilization of sucrose and inulin on a genetic level correlates with the mycelial weight results originated form liquid cultures (Figure 3A).

The expression level of a set of 481 plant biomass utilization-related genes, including 217 CAZy, 168 metabolic genes, 85 transporter genes and 11 TF genes were analyzed to evaluate the involvement of both TFs in the regulation of sucrose and inulin utilization in more detail. As expected, the deletion of *inuR* showed the highest individual impact on CAZy, metabolic and transporter gene expression when grown on the test substrates (Figure 4B, Data S1). A large number of plant biomass utilization-related genes were downregulated in the Δ*inuR* mutant at both timepoints on sucrose (72 and 71 genes after 2 and 8 h of growth, respectively), while only 8 and 46 genes were downregulated on inulin after 2 and 8 h of growth, respectively (Figure 4B).

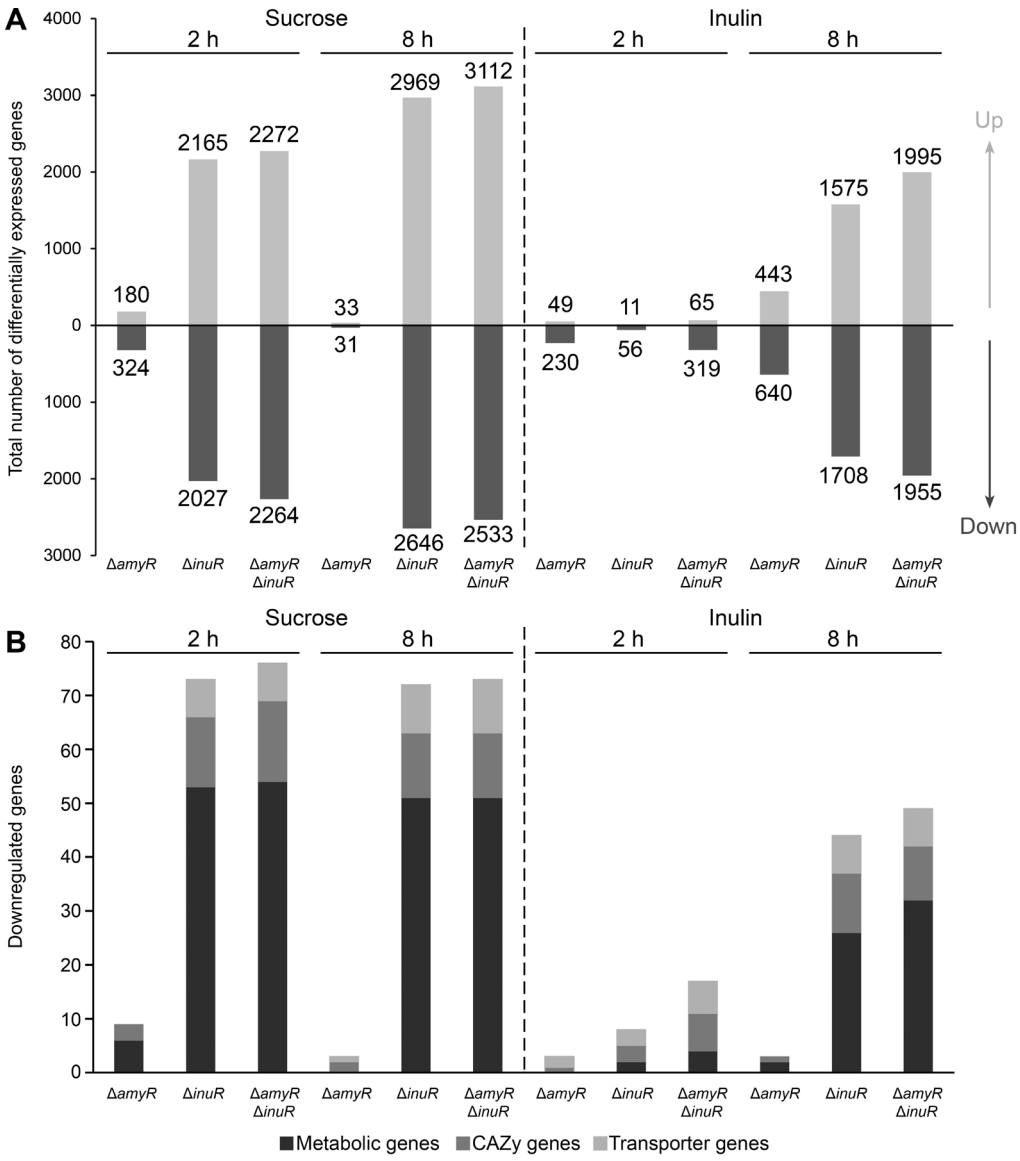


Figure 4. Differentially expressed genes in *A. niger* Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* liquid cultures compared to the control (CBS 138852). Samples originated from 2 or 8 h of growth in liquid minimal medium containing 1% sucrose or 1% inulin as carbon source. **A**) Total number of differentially expressed genes across the genome. Up- or downregulated genes are indicated by different tints of grey. **B**) Downregulated plant biomass degradation-related genes. CAZy, metabolic and transporter genes are indicated by different tints of grey.

Therefore, only a small set of 7 genes were affected by the deletion of *inuR* in all four experimental conditions (Table 1). These include *sucA/suc1*, *inuE/inu1*, *mstH* and the putative maltose/sucrose transporter gene NRRL3_3594[36]. Based on our results, an additional putative inositol/fructose transporter gene, NRRL3_11807[36] is controlled by InuR. The expression of *aglC* was substantially reduced in the Δ*inuR* strain compared to the control. Similarly, reduced expression of *aglC* was observed in a Δ*amyR* mutant when grown on maltose and starch, but not on D-glucose[8], which was shown to induce the expression of *aglC*[37]. Therefore, in our study, the reduced expression of *aglC* in the Δ*inuR* strain is most likely related to impaired release of D-glucose from sucrose and inulin. However, the dependency of *aglC* expression on D-glucose concentration is not fully understood. The previously reported InuR dependent genes, *inuA* and *sucB*[22], were also downregulated in the Δ*inuR* mutant. However, these genes showed generally low expression (FPKM < 20) in the control strain and were excluded from the analysis (Data S1). Interestingly, the invertase gene *sucC*, was not expressed in any of our test conditions (Table S1).

Table 1. Downregulated genes in the *A. niger* Δ*inuR* strain compared to the control. The genes included in this table were downregulated on both media containing 1% sucrose or 1% inulin after 2 and 8 h of growth. Gene expression is represented as FPKM values.

Gene number	Gene name	Description	Reference	Sucrose				Inulin			
				Control 2h	Δ*inuR* 2h	Control 8h	Δ*inuR* 8h	Control 2h	Δ*inuR* 2h	Control 8h	Δ*inuR* 8h
NRRL3_11752	*sucA/ suc1*	SUC (invertase/β-fructo-furanosidase)	[35]	1606.86	0.25	218.88	2.79	220.34	0.16	312.10	0.48
NRRL3_11807	-	putative inositol/fructose transporter	[36]	376.17	51.66	235.22	22.16	572.01	21.53	1474.03	29.23
NRRL3_16	*aglC*	AGL (α-1,4-galactosidase)	[37]	469.34	25.44	30.79	3.91	127.91	15.72	97.77	2.57
NRRL3_3087	*inuE/ inu1*	INX (exo-inulinase)	[38]	1716.50	1.78	168.76	2.85	1352.05	1.41	1681.95	1.24
NRRL3_3594	-	putative maltose/ sucrose transporter	[36]	84.89	12.34	44.87	3.97	134.79	9.96	170.26	5.79
NRRL3_3879	*mstH*	high-affinity glucose transporter	[39]	662.64	60.42	579.77	17.08	385.58	49.97	1084.95	10.74
NRRL3_8073	*pycA*	pyruvate carboxylase	[40]	5154.97	379.43	650.99	292.21	638.20	303.34	1705.41	524.39

In contrast, the deletion of *amyR* did not indicate substantial contribution of AmyR to sucrose and inulin utilization when cultivated in liquid medium. Only three CAZy and six metabolic genes were downregulated in the Δ*amyR* strain compared to the control after 2 h of growth on sucrose, while an even lower number of genes were downregulated in any of the other conditions. Moreover, only a putative glycerol proton symporter gene (NRRL3_817)[36] showed significantly decreased expression in the Δ*amyR*Δ*inuR* strain compared to the Δ*inuR* single deletion mutant after 8 h of growth on inulin.

Overall, gene expression data generated from liquid cultures did not support an involvement of AmyR in sucrose or inulin utilization at the transcriptomic level, which correlates with the mycelial dry weight measurement results from liquid cultures (Figure 3A).

3.5 AmyR contributes to the utilization of sucrose and inulin when *A. niger* is grown on solid media

In order to explain the impact of *amyR* deletion on growth on solid medium containing 1% sucrose or 1% inulin as carbon source (Figure 2, Figure 3B), transcriptomics data were generated from *A. niger* control, Δ*amyR,* Δ*inuR* and Δ*amyR*Δ*inuR* cultures grown on solid agar plates.

Genome wide transcriptome results indicated that AmyR has a minimal impact on sucrose and inulin utilization in the presence of InuR. This was evidenced by the low number of DEGs in the Δ*amyR* strain compared to the control on both substrates (Figure 5A). However, the additional deletion of *amyR* in the Δ*inuR* background strain resulted in further upregulation and downregulation of 554 and 381 genes on sucrose, respectively (Figure 5A). In total, 31 plant biomass utilization-related genes, including 13 CAZy, 14 metabolic and 4 transporter genes, were downregulated in the double deletion strain compared to Δ*inuR* on sucrose. In contrast, a very small number of genes showed downregulation in the double deletion strain compared to the Δ*inuR* on inulin (Figure 5B), which can be associated with the very low expression of *amyR* (3.83 FPKM) in the Δ*inuR* strain on inulin (Data S2). Moreover, mycelial weight measurements did not indicate either significant difference between the Δ*inuR* and Δ*amyR*Δ*inuR* strains on inulin (Figure 3).

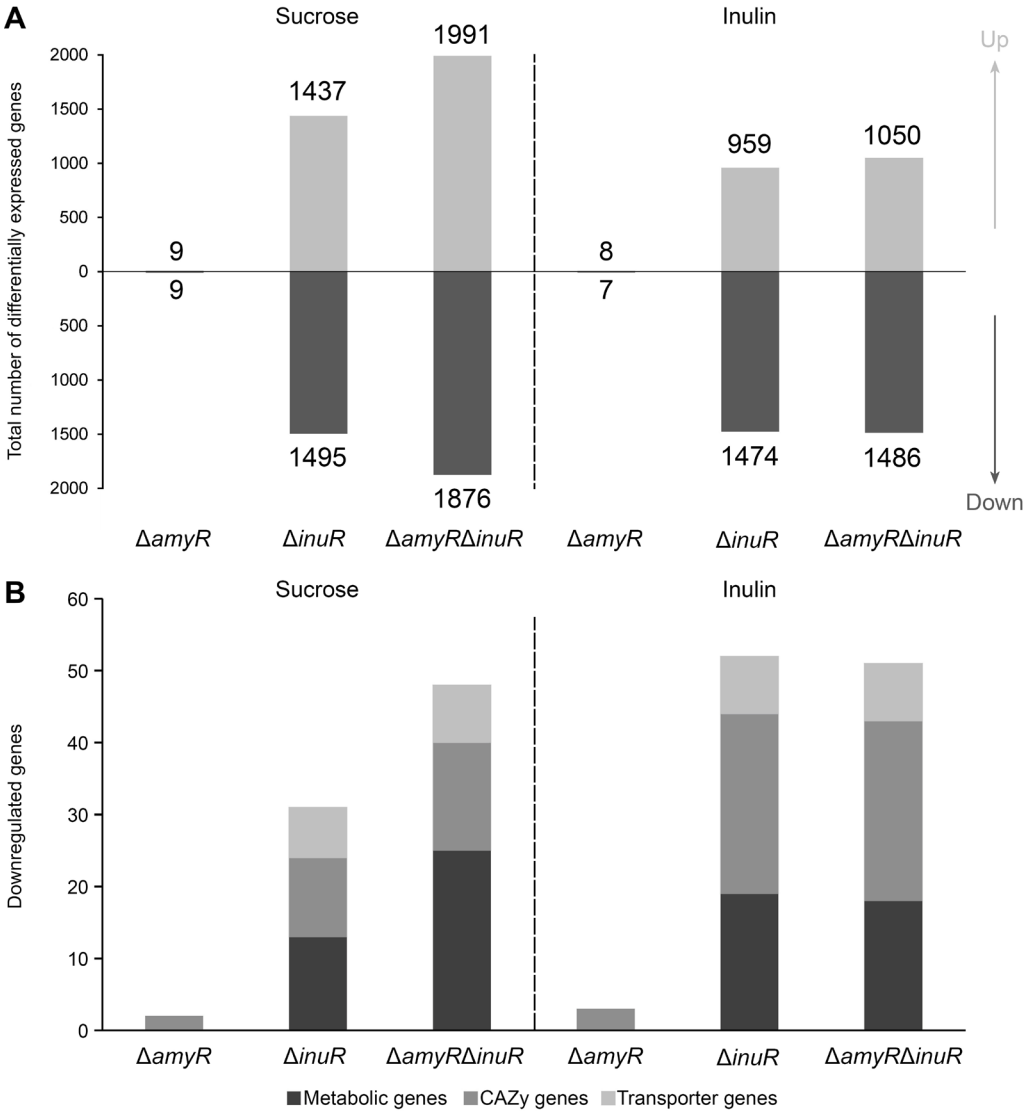


Figure 5. Differentially expressed genes in *A. niger* Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* solid cultures compared to the control (CBS 138852). Samples originated from 40 h of growth on solid minimal medium containing 1% sucrose or 1% inulin as carbon source. **A**) Total number of differentially expressed genes across the genome. Up- or downregulated genes are indicated by different tints of grey. **B**) Downregulated plant biomass degradation-related genes. CAZy, metabolic and transporter genes are indicated by different tints of grey.

The involvement of AmyR in sucrose and inulin utilization is most likely mediated through the regulation of α-glucosidase activity. This activity has been reported to play a role in sucrose utilization[41–43] and can be involved in the removal of terminal α-D-linked glucose units from inulin. A previous study indicated that the expression of α-glucosidase-encoding genes *agdA* and *agdB* was reduced when *amyR* was deleted[22]. In contrast, our results showed increased expression of *agdB* in the Δ*amyR*Δ*inuR* strain compared to the control when grown on solid sucrose and inulin containing medium. In fact, *agdB* was the highest expressed CAZy gene in the Δ*amyR*Δ*inuR* strain cultivated on both solid carbon sources, correlating with previous observations that the expression of this gene is not AmyR dependent[14]. However, the expression of *agdA* was significantly downregulated in both Δ*amyR* and Δ*amyR*Δ*inuR* deletion strains compared to the control when cultivated on solid medium containing sucrose or inulin as carbon source. Moreover, the deletion of *inuR* resulted in the upregulation of *agdA* compared to the control (302.13 FPKM compared to 68.86 FPKM, respectively) (Data S2) on solid medium containing sucrose, most likely to compensate for the reduced expression of invertase-encoding genes (eg., *sucA* and *sucB*)[44]. Therefore, the additional deletion of *amyR* in the Δ*inuR* strain, and the subsequent downregulation of *agdA* (15.13 FPKM) (Data S2) could explain the significant growth reduction of the Δ*amyR*Δ*inuR* strain compared to Δ*inuR* on sucrose (Figure 2 and Figure 3B).

Interestingly, on solid medium, the putative endo-arabinanase encoding gene *abnC* was among the highest expressed CAZy genes in the Δ*inuR* and Δ*amyR*Δ*inuR* strains grown on inulin, as well as in the Δ*amyR*Δ*inuR* strain grown on sucrose, but not in the Δ*inuR* strain grown on the same substrate. The high expression of this gene could be associated with stress or starvation, as it was also observed to show high expression values (along with the putative exo-galactanase encoding gene NRRL3_10498 and the putative endo-arabinanase encoding gene NRRL3_3855) on a complex crude substrate, sugar beet pulp, when the utilization of major components was blocked by the deletion of key TFs[32]. In contrast, in liquid cultures, besides *abnC*, both NRRL3_10498 and NRRL3_3855 were the highest expressed CAZy genes in the Δ*inuR* and Δ*amyR*Δ*inuR* strains when grown on sucrose or inulin for 8 h. This indicates signs of stress or starvation in liquid medium, even in the Δ*inuR* strain that retains the *amyR* gene. This data further supports some involvement of AmyR in the utilization of sucrose and inulin in liquid media and its role in the utilization of sucrose on solid plate cultures.

4. Conclusions

In conclusion, the analysis of data originated from liquid culture samples indicated no involvement of AmyR in sucrose and inulin utilization. However, growth profiling, analysis of mycelial dry weight and transcriptome data originated from solid medium cultures indicated substantial involvement of AmyR in the regulation of sucrose utilization, as well as in the regulation of inulin utilization to a limited extent. Our data show that the amylolytic regulator AmyR is partially involved in the utilization of sucrose and inulin in *A. niger*, by controlling the expression of α-glucosidase genes. In particular, the expression of the AmyR-dependent gene *agdA* correlates with the ability of *A. niger* to grow on sucrose and inulin when the major inulinolytic regulator, InuR, is deleted. In contrast, *agdB* does not show an AmyR dependent expression, and the residual growth of Δ*amyR*Δ*inuR* strain observed on sucrose and inulin could be associated with the activity of AgdB.

Overall, these results show that submerged cultures, which are most often used in laboratory and industrial setups do not always reflect the role of TFs in the natural growth conditions of the fungus, which is rather represented by growth on solid substrates[25].

Data availability

The raw RNAseq data originated from liquid culture samples were deposited at the Sequence Read Archive at NCBI with sample accession numbers SRP379898-379907, SRP379909-379920 and SRP379922-379945. The raw RNAseq data originated from solid culture samples were deposited to GEO with accession ID GSE204768. All other data are available in the main text or in the supplementary files.

CRediT authorship contribution statement

Roland S. Kun: Investigation, Formal analysis, Visualization, Writing – original draft. Sonia Salazar-Cerezo: Investigation, Formal analysis, Writing – original draft. Mao Peng: Formal analysis. Yu Zhang: Investigation, Formal analysis. Emily Savage: Investigation, Formal analysis. Anna Lipzen: Data curation, Formal analysis. Vivian Ng: Project administration. Igor V. Grigoriev: Supervision. Ronald P. de Vries: Conceptualization, Resources, Funding acquisition, Supervision, Writing – review & editing. Sandra

Garrigues: Investigation, Formal analysis, Supervision, Writing – review & editing.

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Supporting information

All supporting information are available upon request from the author.

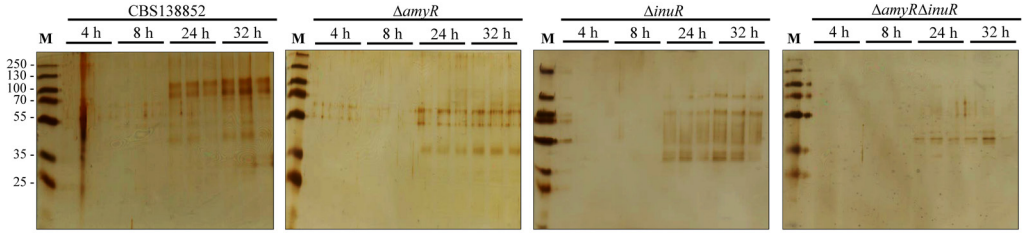

Figure S1. Protein production analysis of *A. niger* parental, Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* strains. SDS-PAGE analysis of the cell-free supernatants after 4, 8, 24 and 32 h of growth on MM + 1% inulin. Ten µL of supernatant were loaded per well. Three biological samples are shown per strain. M: PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific).

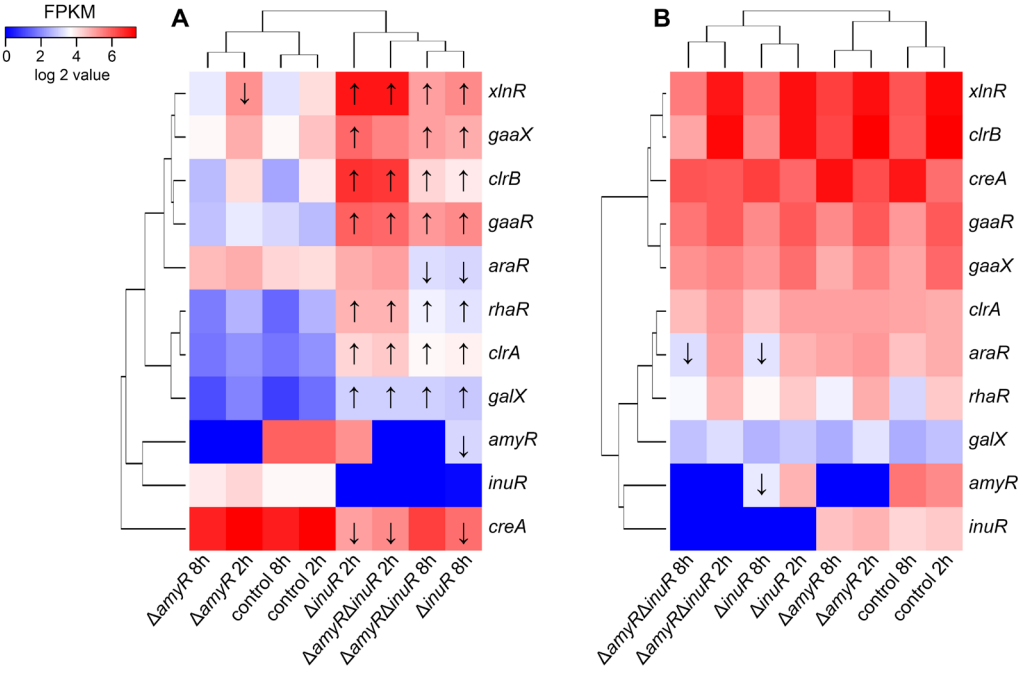

Figure S2. Hierarchical clustering of transcription factor genes in *A. niger* control (CBS 138852) and Δ*amyR*, Δ*inuR* and Δ*amyR*Δ*inuR* deletion mutants. Data originated from 2 and 8 h of culturing in 1% sucrose (**A**) or 1% inulin (**B**) liquid minimal medium. Downregulated genes (fold change < 0.5; *p*adj < 0.01) in the deletion mutants compared to the control are indicated by a downward arrow. Upregulated genes (fold change > 2; *p*adj < 0.01) are indicated by an upward arrow. 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.

Table S1. *A. niger* strains used in this study.

Table S2. Primers used in this study.

Data S1. Gene expression values of CAZy-, metabolic-, sugar transporter- and transcription factor genes of *A. niger* control (CBS 138852) and deletion mutant strains cultivated in liquid medium. Samples originate from 1% sucrose or inulin liquid cultures after 2 and 8 h of growth. Gene expression values of FPKM < 20 were considered low, and are indicated in red. Upregulated genes (fold change > 2) are indicated in blue, while downregulated genes (fold change < 0.5) are indicated in orange. Statistically significant changes (*p*adj < 0.01) are indicated in green.

Data S2. Gene expression values of CAZy-, metabolic-, sugar transporter- and transcription factor genes of *A. niger* control (CBS 138852) and deletion mutant strains cultivated on solid medium. Samples originate from 1% sucrose or inulin solid cultures after 40 h of growth. Gene expression values of FPKM < 20 were considered low, and are indicated in red. Upregulated genes (fold change > 2) are indicated in blue, while downregulated genes (fold change < 0.5) are indicated in orange. Statistically significant changes (*p*adj < 0.01) are indicated in green.


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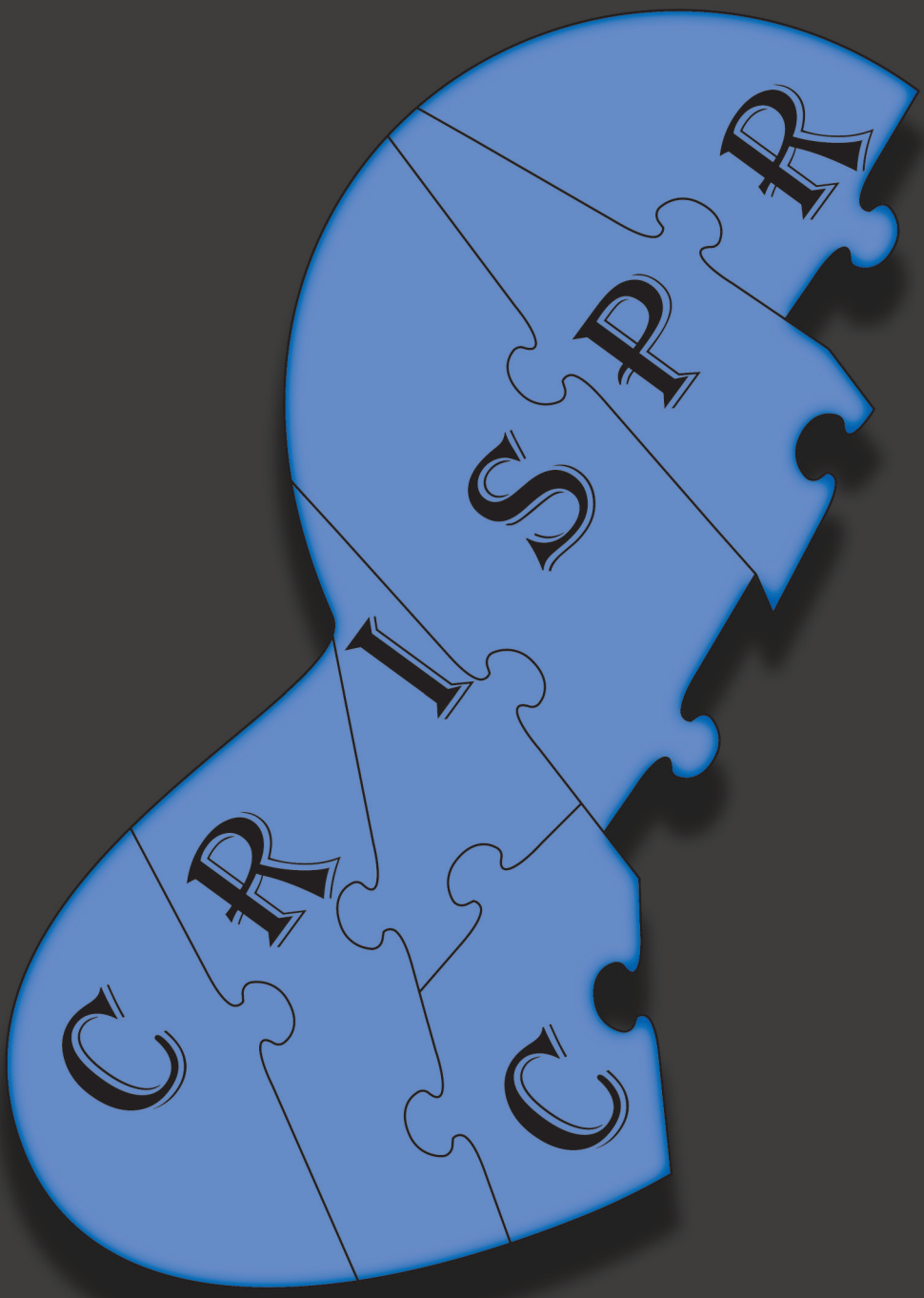
CRISPR

Chapter 7

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

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Abstract

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 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.

1. Introduction

Plant biomass is the most abundant carbon source on earth and mainly consists of plant cell wall polymers (cellulose, hemicellulose, pectin and lignin)[1]. 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[2]. In this context, low-cost substrates are of high interest for many biotechnological applications. Wheat bran, a byproduct 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[3–7].

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[8].

The first identified (hemi-)cellulolytic regulator is the *A. niger* XlnR[9], and orthologs have been widely studied in other fungal species[8], 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[10], 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[11]. 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[12]. So far, CLR-1 homologs showing conserved function have been reported for *A. nidulans*[12] and *A. niger*[13] (ClrA) while CLR-2 homologs have been described for multiple species, such as *A. nidulans*[14], *A. niger*[13], *Penicillium oxalicum*[15] (ClrB), and *A. oryzae*[16]. In *A. oryzae* the CLR-2 homolog was initially described as a regulator of β-mannan utilization (ManR)[17], 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 to ClrA and both appear to be influenced

by XlnR[13]. 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[18] (starch degradation), InuR[19] (inulin degradation) and GaaR[20], RhaR[21] and GalX[22] (pectin degradation) are expected to have no or a minor role.

In this study, we used CRISPR/Cas9 genome editing[23] 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.

2. Results

2.1 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 (Figure 1A and Figure 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 (Figure 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 to the control strain was observed in the other mutant strains.

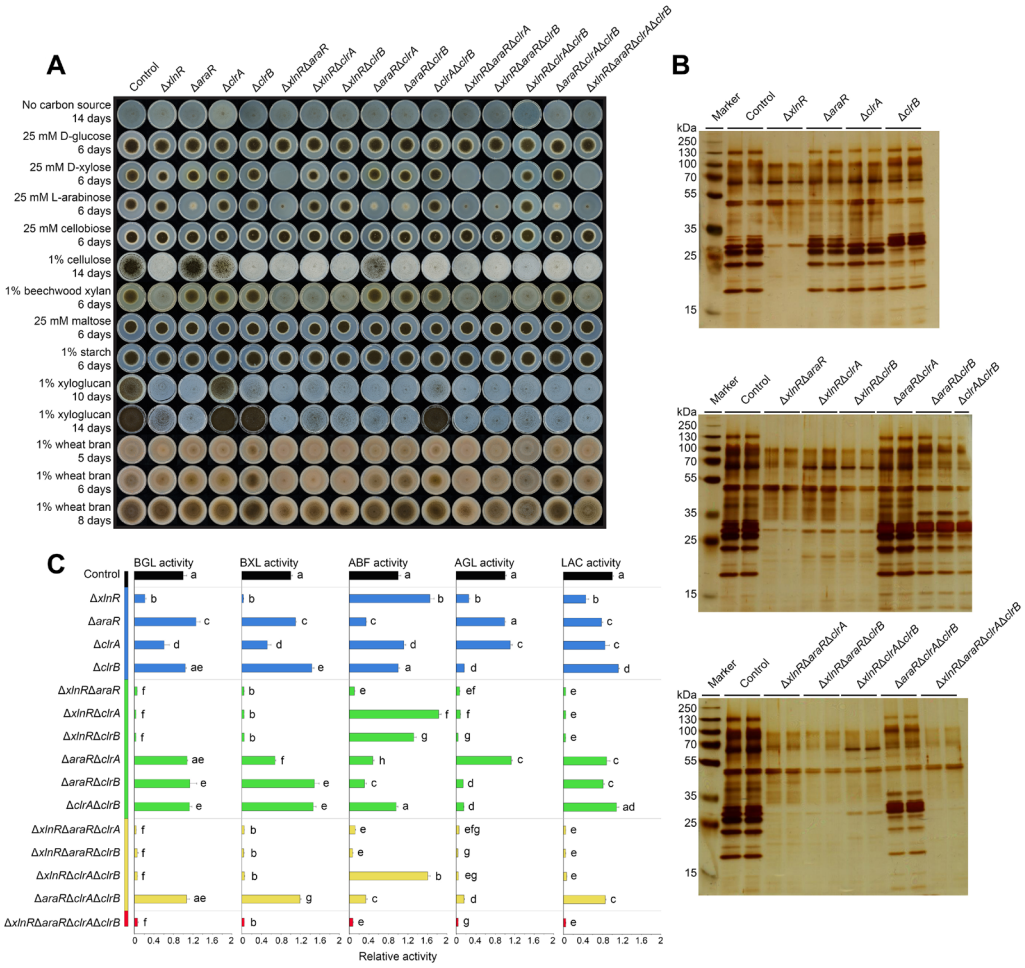


Figure 1. Characterization of *A. niger* XlnR-AraR-ClrA-ClrB deletion mutants. A) Growth profile of *A. niger* control and mutant strains. Selected carbon sources were inoculated with 1000 spores and incubated at 30°C for up to 14 days. **B**) Extracellular protein production of *A. niger* control and 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, $p < 0.05$).

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.

2.2 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 (Figure 1A) could be a direct result of reduced enzyme levels, samples of liquid cultures containing 3% wheat bran were first analysed by SDS-PAGE (Figure 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 (Figure 1A) correlates with the reduction of β-1,4-D-glucosidase (BGL) activity in this strain (Figure 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 than XlnR in *A. niger*. The abolished growth on cellulose of Δ*clrB* cannot be explained by reduced BGL activity, suggesting that ClrB is more important for the production of cellobiohydrolases and/or endoglucanases[13], 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 catalyzed by β-xylosidase (BXL) activity. Similar to BGL activity, deletion of *xlnR* showed the highest impact on BXL activity (Figure 1C), which was abolished in every mutant in which *xlnR* was deleted, and which correlated with minimal growth of these strains on beechwood xylan (Figure 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) (Figure 1C) and the growth profile (Figure 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 of 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.

2.3 Residual starch in wheat bran explains the limited growth reduction of 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 (Figure 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*.

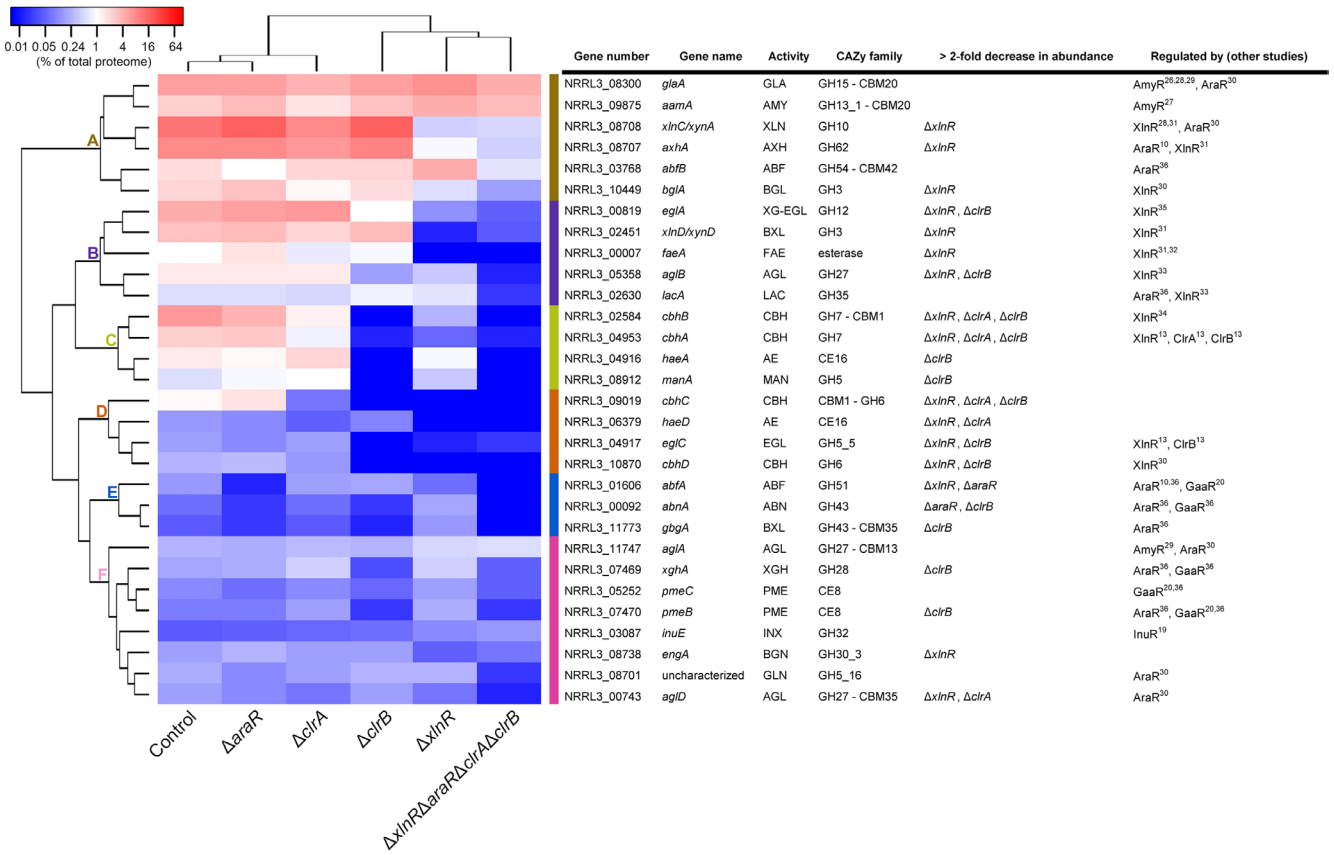
Of the single deletion mutants, Δ*xlnR* showed the highest reduction of CAZymes, which is also reflected in the SDS-PAGE (Figure 1B) and enzyme activity (Figure 1C) results. The minimal presence of (arabino)xylan-acting enzymes in the Δ*xlnR* supernatant was similar to that of the quadruple mutant, although the overall abundance of cellulolytic enzymes was further reduced in the supernatant of the quadruple mutant (Figure 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)[24] in their promoter sequences (Table S2), thus suggesting direct regulation of these genes by XlnR.

The quadruple deletion mutant showed further reduction of the abundance of two cellobiohydrolases (CbhA and CbhB), an acetylesterase (HaeA) and a β-1,4-endo-mannanase (ManA) compared to the Δ*xlnR* mutant (Figure 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 ($CGGN_8CCG$)[25] (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 to any single deletion strain (Figure 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 two-fold (Figure 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[26–30].

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 (Figure 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 to the quadruple mutant (Figure S2). This result demonstrates the ability of *A. niger* to maintain growth by using the starch component of wheat bran, when utilization of the major carbohydrates is blocked.



Gene number	Gene name	Activity	CAZy family	> 2-fold decrease in abundance	Regulated by (other studies)
NRRL3_08300	*glaA*	GLA	GH15 - CBM20		AmyR[26,28,29], AraR[30]
NRRL3_09875	*aamA*	AMY	GH13_1 - CBM20		AmyR[27]
NRRL3_08708	*xlnC/xynA*	XLN	GH10	Δ*xlnR*	XlnR[28,31], AraR[30]
NRRL3_08707	*axhA*	AXH	GH62	Δ*xlnR*	AraR[10], XlnR[31]
NRRL3_03768	*abfB*	ABF	GH54 - CBM42		AraR[36]
NRRL3_10449	*bglA*	BGL	GH3	Δ*xlnR*	XlnR[30]
NRRL3_00819	*eglA*	XG-EGL	GH12	Δ*xlnR*, Δ*clrB*	XlnR[35]
NRRL3_02451	*xlnD/xynD*	BXL	GH3	Δ*xlnR*	XlnR[31]
NRRL3_00007	*faeA*	FAE	esterase	Δ*xlnR*	XlnR[31,32]
NRRL3_05358	*aglB*	AGL	GH27	Δ*xlnR*, Δ*clrB*	XlnR[33]
NRRL3_02630	*lacA*	LAC	GH35		AraR[36], XlnR[33]
NRRL3_02584	*cbhB*	CBH	GH7 - CBM1	Δ*xlnR*, Δ*clrA*, Δ*clrB*	XlnR[34]
NRRL3_04953	*cbhA*	CBH	GH7	Δ*xlnR*, Δ*clrA*, Δ*clrB*	XlnR[13], ClrA[13], ClrB[13]
NRRL3_04916	*haeA*	AE	CE16	Δ*clrB*	
NRRL3_08912	*manA*	MAN	GH5	Δ*clrB*	
NRRL3_09019	*cbhC*	CBH	CBM1 - GH6	Δ*xlnR*, Δ*clrA*, Δ*clrB*	
NRRL3_06379	*haeD*	AE	CE16	Δ*xlnR*, Δ*clrA*	
NRRL3_04917	*eglC*	EGL	GH5_5	Δ*xlnR*, Δ*clrB*	XlnR[13], ClrB[13]
NRRL3_10870	*cbhD*	CBH	GH6	Δ*xlnR*, Δ*clrB*	XlnR[30]
NRRL3_01606	*abfA*	ABF	GH51	Δ*xlnR*, Δ*araR*	AraR[10,36], GaaR[20]
NRRL3_00092	*abnA*	ABN	GH43	Δ*araR*, Δ*clrB*	AraR[36], GaaR[36]
NRRL3_11773	*gbgA*	BXL	GH43 - CBM35	Δ*clrB*	AraR[36]
NRRL3_11747	*aglA*	AGL	GH27 - CBM13		AmyR[29], AraR[30]
NRRL3_07469	*xghA*	XGH	GH28	Δ*clrB*	AraR[36], GaaR[36]
NRRL3_05252	*pmeC*	PME	CE8		GaaR[20,36]
NRRL3_07470	*pmeB*	PME	CE8	Δ*clrB*	AraR[36], GaaR[20,36]
NRRL3_03087	*inuE*	INX	GH32		InuR[19]
NRRL3_08738	*engA*	BGN	GH30_3	Δ*xlnR*	
NRRL3_08701	uncharacterized	GLN	GH5_16		AraR[30]
NRRL3_00743	*aglD*	AGL	GH27 - CBM35	Δ*xlnR*, Δ*clrA*	AraR[30]

Figure 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[10,13,32–36,19,20,26–31]. 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.

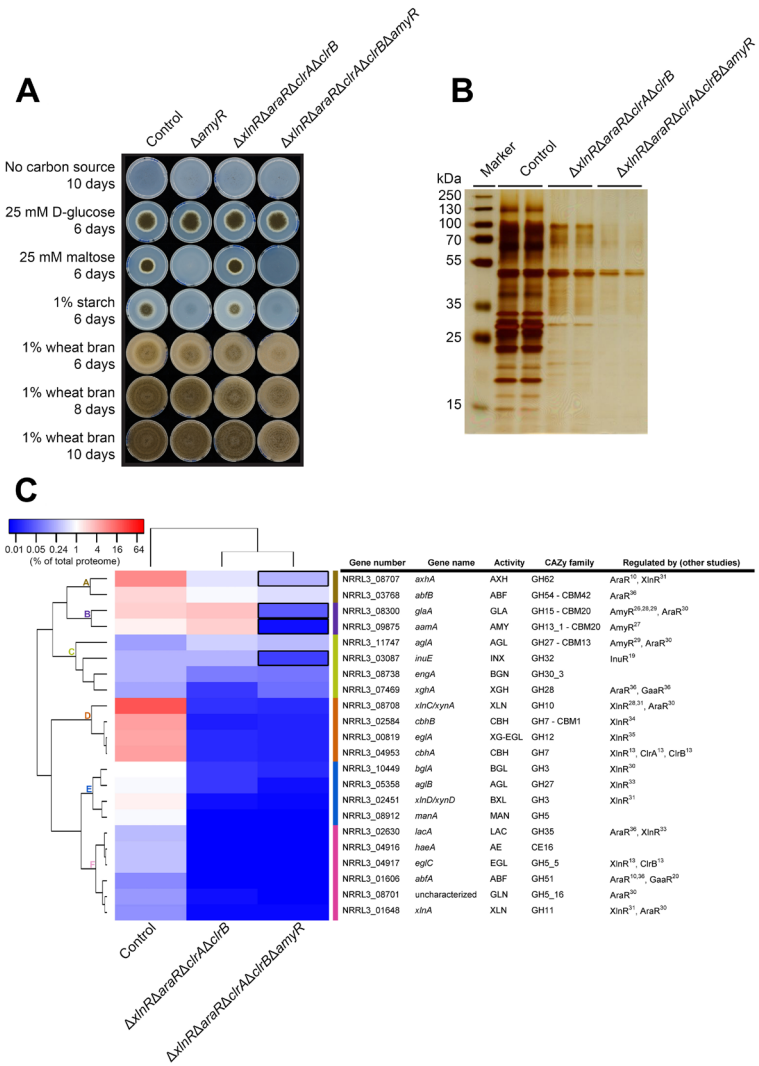


Figure 3. Characterization of *A. niger* Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* quintuple deletion mutant. A) Growth profile of *A. niger* control, and the Δ*amyR*, Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* and Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* mutant strains. Selected carbon sources were inoculated with 1000 spores 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 >2-fold decrease in abundance compared to the Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* strain. Regulation of genes is based on previous studies[10,13,33–36,19,20,26–31]. 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.

2.4 The Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* 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 to the quadruple strain due to the lack of amylolytic enzymes (Figure 3B). The CAZyme content in the supernatant of the quintuple mutant (Figure 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 (Figure 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 (Figure 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 (Figure 3A). Interestingly, the abundance of an arabinoxylan arabinofuranohydrolase (AxhA) (Figure 3C, cluster A) and an exo-inulinase (InuE) (Figure 3C, cluster C) was also reduced more than two-fold compared to 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 (Figure 4A and Data S3B). In all strains, the most abundant non-CAZy protein was the aspartic peptidase PepA (Figure 4B, cluster A), which is under the control of the transcription factor PrtT, a specific regulator of extracellular proteases in filamentous fungi[37]. PepA, together with other PrtT-controlled proteases (ProtA, ProtB, NRRL3_11745, PepF, NRRL3_05873 and NRRL3_01776)[38] showed a relative increase in abundance when the (hemi-)cellulolytic enzyme system was impaired in the quadruple and quintuple deletion mutants (Figure 4B and Data S2A and S3A). Lipases were detected in very small amounts, with only one (Lipanl) detected at > 0.1% abundance in all strains (Figure 4B, cluster B), but with its highest abundance (1.95%) in the quintuple mutant (Data S3A). Overall, these results indicate that the quintuple mutant utilizes proteins as a primary carbon source when carbohydrate catabolism is blocked.

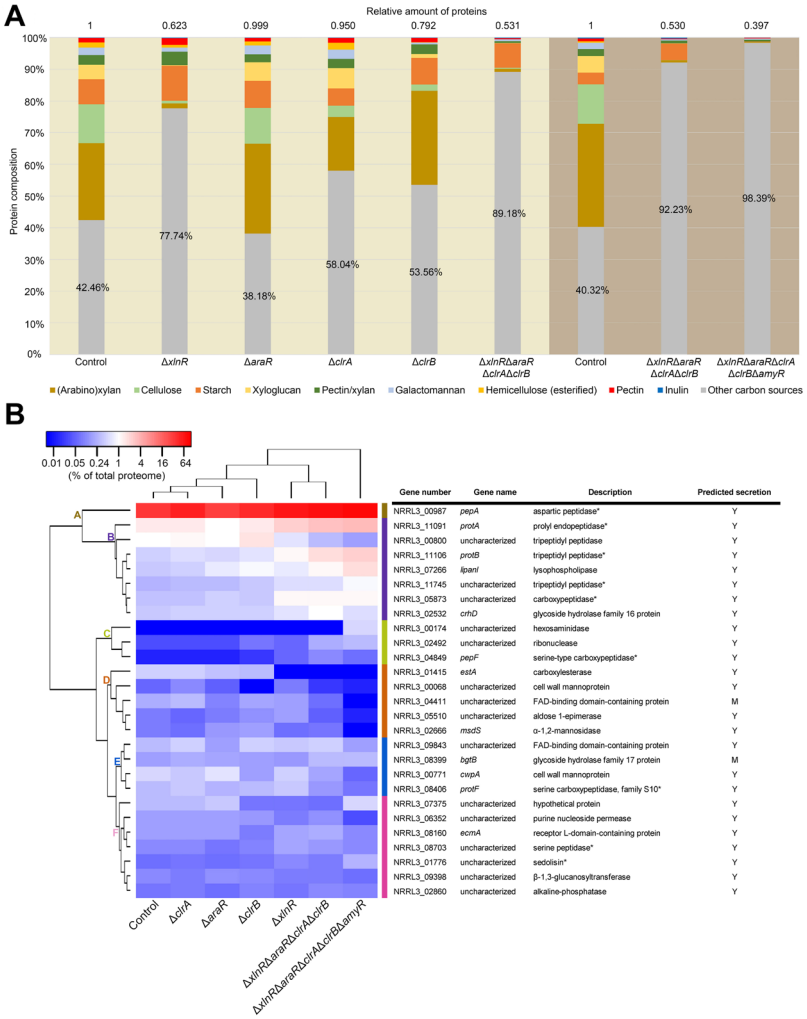


Figure 4. Analysis of non-CAZyme proteins produced by *A. niger* control and deletion mutant strains. A) Relative composition of the total exoproteome of *A. niger* control and mutant strain supernatant samples originated from 3% wheat bran after 24 h. CAZymes are classified based on the substrates they 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 proteomics 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 proteomics data set (Data S2A), while the Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* data originates from the second proteomics data set (Data S3A).

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.

3. 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)[39,40]. However, efficient design of such cell factories requires a detailed understanding of the plant biomass conversion process at the molecular level[41]. 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 suggest 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[3,42]. 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[43]. 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[44]. However, a comparative study of the *Trichoderma reesei* and *Talaromyces cellulolyticus* (formerly *Acremonium cellulolyticus*) secretomes showed differences in the major enzymatic activities[45]. The supernatant derived from the *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*. Among others, the synergistic action of xylanases and cellulases is necessary for the enzymatic degradation of various agricultural residues or woody substrates for biofuel production[46].

The phenotype and exoproteome of the *A. niger* Δ*xlnR* mutant confirmed previous studies that reported impaired growth on cellulose, (arabino)xylan and xyloglucan[30,31]. Enzyme activity assays and proteomics 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[47]. 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[13]. 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 conclude that BGL and BXL encoding genes are controlled by XlnR and ClrA in *A. niger*. Furthermore, our proteomics 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 to ClrA in *A. niger*[13], 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[25] 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[1]. 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[10].

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*[36]. In our case, none of the single (hemi-)cellulolytic transcription factor deletion mutants showed strong reduction of 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[48]. The strong reduction in growth of the quintuple mutant indicated that starch is responsible for the only small growth reduction of 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[29]. However, the quintuple mutant still showed residual growth on wheat bran, most likely by utilizing proteins and other minor components, such as lipids[49,50]. 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[37,38]. The major PrtT-controlled proteases (PepA, ProtA and ProtB) represented a relatively higher proportion of the exoproteome in the quintuple mutant compared to 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[38]. However, it is more likely that this is a starvation-induced response as utilization of the major carbohydrates is blocked in the quintuple mutant and starvation has been shown to cause protease induction[51]. 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 shows 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 (Figure 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 (Figure 5B).

Figure 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 >2-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-L-arabinofuranosidase (AbfA), one β-D-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-endo-glucanase (EngA), one β-1,4-endo-glucanase (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 of the corresponding deletion mutants compared to the control. Relative growth has been estimated after 6 days of incubation at 30°C. No growth difference was observed between biological replicates.

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[50,52]. This hypothesis is supported by the different roles of ClrA and ClrB in *N. crassa*[12], *A. nidulans*[12] and *A. oryzae*[16], compared to *A. niger*, as well as the highly diverse set of target genes of XlnR in different fungi[53]. 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.

4. Experimental procedures

4.1 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 ampicillin (Sigma Aldrich). Fungal strains used in this study were derived from the *A. niger* CBS 138852 (*cspA1*, *pyrG*$^{-}$, *kusA*::*amdS*) strain[54]. 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)[55] supplemented with 1% D-glucose and 1.22 g/L 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[42]. Washing was performed by autoclaving wheat bran at 5% concentration in demineralized MilliQ water. After autoclaving, the medium was centrifuged at 1800 x *g* for 10 min. The supernatant was removed and the insoluble wheat bran pellet was resuspended in sterile demineralized MilliQ 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 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.

For liquid cultures, freshly harvested spores were pre-grown in 250 mL CM containing 2% D-fructose and 1.22 g/L uridine for 16 h at 30°C in a rotary shaker at 250 rpm. After 16 hours, mycelia were harvested by filtration through sterile cheesecloth, rinsed with MM, and approximately 2.5 g (wet weight) mycelium was transferred into 50 mL MM containing 3% wheat bran. Supernatant samples were taken after 24 h 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.

4.2 Construction of mutant strains

The ANEp8-Cas9-*pyrG* plasmid[23], which contains the autonomous fungal replicating element AMA1[56], *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[57]. 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[58]. 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, Inc. (IDT, Leuven, Belgium) and are shown in Table S5.

4.3 SDS-PAGE and enzyme activity assays

Cell-free supernatant samples of 3% wheat bran liquid cultures were harvested after 24 h incubation at 30°C in a rotary shaker at 250 rpm. Twelve µL 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[58]. Enzyme activities were evaluated based on colorimetric *p*-nitrophenol (*p*NP) 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, AGL activity was measured after 15 min, while AGD and GLA activities were measured after 20 h 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.

4.4 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).

4.5 Proteomics 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 × *g*, 4°C for 20 min. The supernatant was aspired 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 (BioRad, Mississauga, Ontario). Five µg of protein samples of biological duplicates were digested with trypsin for proteomic analysis as previously described[59]. Dried peptide digest samples were solubilized in a solution of 5% acetonitrile, 0.1% formic acid and 4 fmol/µL of trypsin-digested Bovine Serum Albumin (BSA) (Michrom, Auburn, CA) used as internal standard. Five µl 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 was 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 R 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.

4.6 Binding site analysis

Binding site analysis for the target transcription factors was performed using the RSAT online tool[60] (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'[24] and 5'-CGGNTAAW-3'[61] for XlnR, 5'-CGGDTAAW-3'[61] for AraR, 5'-CGGN$_8$CCG-3'[25] for ClrB and 5'-CGGN$_8$CGG-3'[18] for AmyR were analysed in this study.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[62] partner repository with the dataset identifier PXD023338 and 10.6019/PXD023338 (http://www.ebi.ac.uk/pride/archive/projects/PXD023338). All other data are available in the main text or in the supplementary files.

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 proteomics analysis. A.T. and R.P.dV. designed the experiments, supervised the research, reviewed and edited the manuscript.

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Supporting information

All supporting information are available at: https://doi.org/10.1111/1751-7915.13835 or can be obtained upon request from the author.

Figure 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.

Figure 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.

Figure 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.

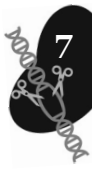
Table S6. Summary of the ANOVA analysis for each enzymatic assay.

Table S7. Enzyme abbreviations used in this study.

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 (Figure 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 (Figure 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.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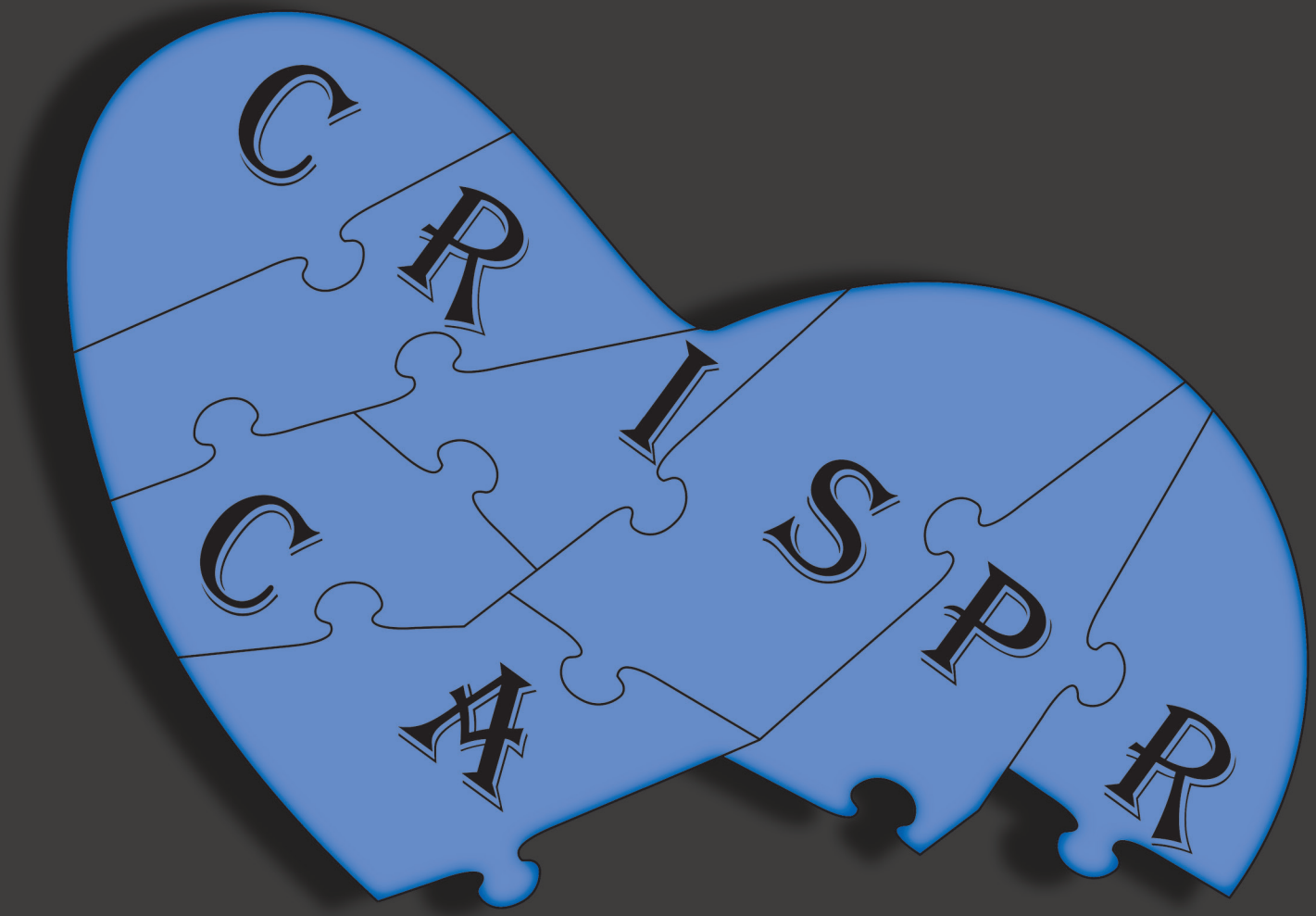
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Chapter 8

Unraveling the regulation of sugar beet pulp utilization in the industrially relevant fungus *Aspergillus niger*

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Abstract

Efficient utilization of agro-industrial waste, such as sugar beet pulp, is crucial for the biobased economy. The fungus *Aspergillus niger* possesses a wide array of enzymes that degrade complex plant biomass substrates, and several regulators have been reported to play a role in their production. The role of the regulators GaaR, AraR and RhaR in sugar beet pectin degradation has previously been reported. However, genetic regulation of the degradation of sugar beet pulp has not been assessed in detail. In this study, we generated a set of single and combinatorial deletion mutants targeting the pectinolytic regulators GaaR, AraR, RhaR and GalX as well as the (hemi-)cellulolytic regulators XlnR and ClrB to address their relative contribution to the utilization of sugar beet pulp. We show that *A. niger* has a flexible regulatory network, adapting to the utilization of (hemi-)cellulose at early timepoints when pectin degradation is impaired.

1. Introduction

Plant biomass is the most abundant renewable terrestrial resource, and is considered a valuable raw material for an increasing number of biotechnological applications such as pulp and paper, food and feed, textiles, detergents, biofuels and biochemicals[1]. It mainly consists of plant cell wall polysaccharides (cellulose, hemicellulose, pectin), lignin, proteins, and storage polysaccharides (starch, inulin, and gums)[2]. Achieving an efficient use of plant biomass as feedstock is crucial in the current bio-based economy scenario of valorizing renewable resources. In this context, low-cost plant biomass substrates are of high interest.

Sugar beet pulp is the main by-product of industrial sugar beet (*Beta vulgaris*) processing, and is currently sold as low-value animal feed. Only in USA, around 30 million tons of sugar beets are produced annually, generating over 1.5 million tons of sugar beet pulp as dry residue[3]. It has been reported that 1 ton of sugar beet yields approximately 150 kg of sugar and 500 kg of wet beet pulp (or 210 kg pressed beet pulp, or 50 kg dehydrated beet pulp)[4]. Degradation of sugar beet pulp polymeric carbohydrates into monosaccharides is a promising step toward increasing the value of this by-product of the sugar industry. This substrate is especially rich in cellulose (20-24%), hemicellulose (25-36%) (mainly xyloglucan), and pectin (15-25%)[5], of which the structure has been well-studied[1].

Filamentous fungi, particularly ascomycetes and basidiomycetes, are highly efficient degraders of plant biomass[6,7]. They secrete large amounts or hydrolytic and oxidative enzymes to efficiently degrade the complex structure of plant material[8,9]. These enzymes have been catalogued in the Carbohydrate Active enZyme (CAZy) database (www.cazy.org) in several families and subfamilies according to amino acid sequence similarity and enzymatic activities[10]. Filamentous fungi control the production of plant polysaccharide-degrading enzymes at the transcriptional level to ensure a space-time balanced and optimized enzyme production. Transcription factors (TFs) are regulatory proteins that activate or repress gene expression by specific binding to conserved motifs in the promoters of their target genes. Several TFs involved in the regulation of plant biomass utilization have been characterized in fungi[11].

Aspergillus niger is a biotechnologically relevant ascomycete with a long history of safe use for the production of enzymes and metabolites[12,13]. This fungus has a great potential for plant biomass degradation[14–16], and is the most commonly used species in industry. In *A. niger,* sugar-specific TFs are activated or repressed by the presence of monomeric sugars or intracellular compounds thereof. The TFs involved in the degradation of the

polysaccharides present in sugar beet pulp (cellulose, xyloglucan and pectin) and the utilization of the resulting monosaccharides are of particular interest for sugar beet pulp valorization. Three transcriptional activators, the galacturonic acid-responsive regulator GaaR, the rhamnose-responsive regulator RhaR, and the arabinanolytic regulator AraR, have already been described in *A. niger*[17–19], and their co-regulation for pectin degradation has been studied in this fungus[20], with GaaR playing the most dominant role. D-galactose is particularly present in xyloglucan, pectin and gums (mainly galacto(gluco)mannans)[21]. In *A. niger,* the galactose-responsive regulator GalX has been reported to play a key role in D-galactose catabolism via the oxido-reductive pathway[22]. Additionally, four other TFs have been reported to be involved in (hemi-) cellulose degradation in this species: the xylanolytic regulator XlnR[23]; AraR, which not only controls pectin and hemicellulose degradation in *A. niger*[20,24] but also the Pentose Catabolic Pathway (PCP) together with XlnR[25]; and the cellulose-responsive regulators ClrA and ClrB[26]. However, ClrB plays a more dominant role in the process of cellulose degradation than ClrA in *A. niger*[15,26], and both have been suggested to be under the control of XlnR[26].

The present work aims to study the relative contribution of a set of TFs – GaaR, AraR, RhaR, GalX, XlnR, and ClrB – to the degradation of sugar beet pulp by *A. niger*. For this purpose, we generated a combination of single and multiple deletion mutant strains in these six regulators, and their phenotype and genetic response on sugar beet pulp were analyzed.

2. Results

2.1 Combined deletion of GaaR, AraR, RhaR, GalX, XlnR and ClrB abolished the growth of *A. niger* on sugar beet pulp

In order to study whether the six TFs chosen in this work (GaaR, AraR, RhaR, GalX, XlnR, and ClrB) affect *A. niger* growth on sugar beet pulp, single and multiple deletion mutants were obtained and their phenotypes on washed sugar beet pulp and related carbon sources were analyzed (Figure 1). Growth on D-glucose was used as an internal control and was similar in all strains.

The Δ*clrB* mutant and all mutant combinations containing *clrB* deletion highly reduced *A. niger* growth on sugar beet pulp. Δ*araR,* Δ*gaaR,* and to a lesser degree Δ*rhaR* mutants showed a slight reduction of growth on sugar beet pulp after 8 days of growth, while

growth of Δ*xlnR* and Δ*galX* was comparable to that of the parental strain. Interestingly, Δ*araR,* Δ*xlnR,* Δ*rhaR,* Δ*galX* and Δ*gaaR* showed a similar growth pattern on sugar beet pulp compared to the reference after 14 days of growth. These results suggest that from the six TFs used in this study, ClrB is the most dominant regulator for sugar beet pulp degradation in *A. niger* solid cultures*,* likely owing to the high cellulose content of this crude substrate. Surprisingly, Δ*gaaR* did not show a differential phenotype on sugar beet pulp compared to that of the reference strain at late timepoints, even though GaaR is the most dominant regulator of pectin, which is one of the main components of sugar beet pulp. In contrast, the pectin-related mutants Δ*gaaR*Δ*araR*Δ*rhaR* and Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* showed reduced growth ability on sugar beet pulp. Finally, the sextuple mutant Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* almost completely abolished *A. niger*'s ability to grow on sugar beet pulp and all tested sugar beet pulp-related carbon sources (except for D-glucose) at all timepoints. In order to reveal the individual contribution of the different regulators on sugar beet pulp degradation, growth was also evaluated on the polymeric and monomeric components of this crude substrate (Figure 1).

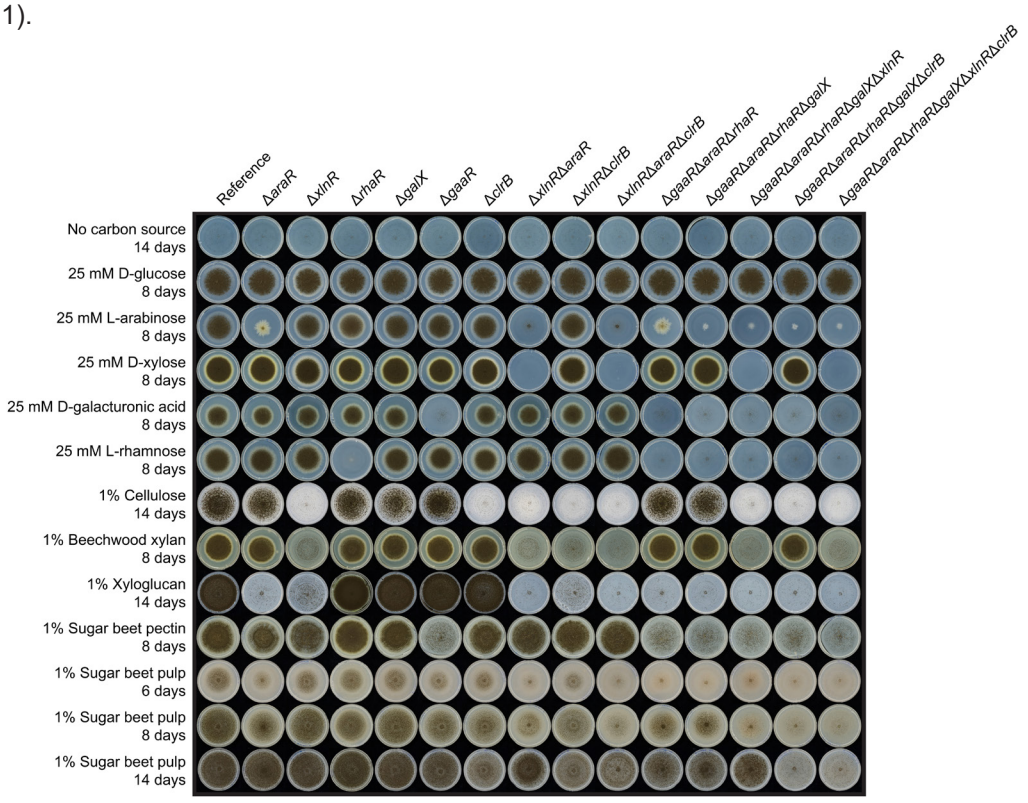


Figure 1. Phenotypic analysis of *A. niger* reference (CBS 138852) and regulatory mutant strains grown on sugar beet pulp and related carbon sources for up to 14 days at 30°C.

No growth was observed for Δ*xlnR* and Δ*clrB* on cellulose, confirming the key role of XlnR and ClrB in cellulose degradation. In addition, Δ*araR,* followed by Δ*xlnR,* showed a strongly reduced growth on xyloglucan, the most abundant hemicellulose present in sugar beet pulp. As expected, Δ*gaaR* showed poor growth on pectin and D-galacturonic acid. Despite D-galactose being an abundant component of pectin, *galX* deletion did not significantly affect growth of *A. niger* on pectin, although its deletion strongly affected growth on L-arabinose in multiple combinatorial deletion mutants (Figure 1). This would suggest a possible involvement of GalX in L-arabinose utilization in this fungus, which is also a major pectin component.

Taking all these results together, abolished growth on sugar beet pulp and all sugar beet pulp components can only be accomplished with the combinatorial deletion of all TFs of this study, suggesting that they all play a role in sugar beet pulp utilization.

2.2 AraR plays a major role in the regulation of extracellular activities in sugar beet pulp liquid cultures

To study the possible correlation between reduced growth ability on sugar beet pulp shown by the different mutant strains (Figure 1) and protein production levels, fungal mycelia were transferred to liquid medium containing 1% washed sugar beet pulp, and cell-free supernatant samples of selected strains grown for 2, 8, and 24 h were analyzed by SDS-PAGE (Figure S1). No protein production could be detected in any of the supernatants after 2 h of incubation on sugar beet pulp (data not shown). Deletion of *araR* or *gaaR* had the highest impact on the overall amount of extracellular proteins produced, which was evident after 8 h of growth. This pattern positively correlates with the growth reduction of Δ*araR* and Δ*gaaR* on sugar beet pulp shown at early timepoints (Figure 1), which disappears after 14 days of growth. In contrast, deletion of *clrB* did not cause a significant change in the protein production pattern compared to that of the reference strain, despite the high impact of *clrB* deletion on growth on sugar beet pulp (Figure 1). All combinatorial mutants showed a highly reduced ability to produce extracellular proteins, with the quadruple Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* and the sextuple Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* mutants showing the same protein production pattern. However, the aforementioned quadruple mutant still showed residual growth ability on sugar beet pulp, whereas the growth of the sextuple mutant was abolished (Figure 1). Assays were performed to evaluate the enzymatic activities present in the exoproteome of the reference and deletion mutant strains (Figure 2).

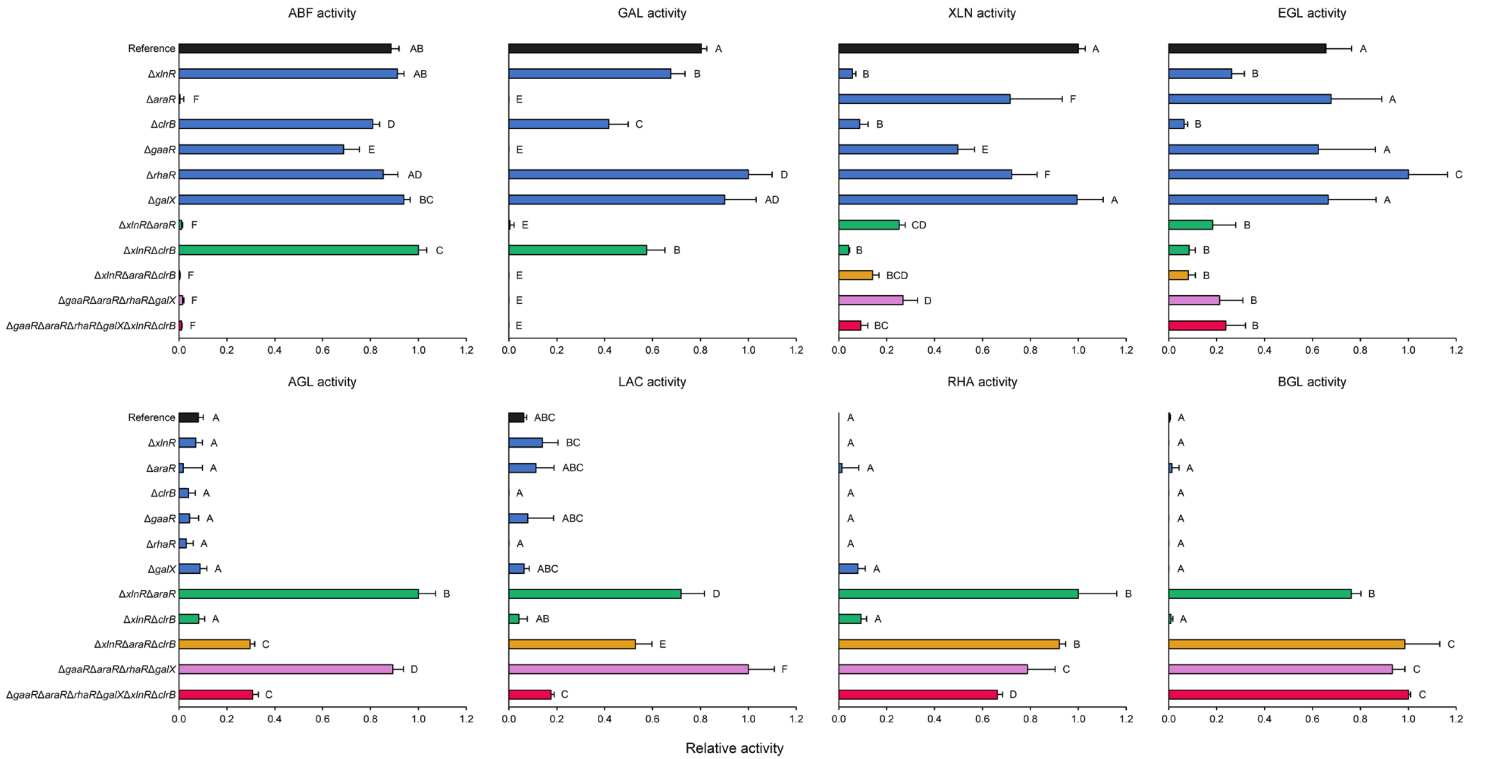


Figure 2. Enzyme activity assays of supernatants from *A. niger* (CBS 138852) reference and deletion mutant strains. The reference, and the selected single-, double-, triple-, quadruple- and sextuple deletion mutant strains are indicated by different colours. Data represent the normalized mean activity values of biological duplicates and technical triplicates and the standard deviation. The raw absorbance values measured at 405 or 600 nm wavelength and the calculated activity values are indicated in Data S1. ABF = α-L-arabinofuranosidase, GAL = endo-1,4-β-galactanase, XLN = endo-1,4-β-xylanase, EGL = endo-1,4-β-glucanase, AGL = α-1,4-D-galactosidase, LAC = β-1,4-D-galactosidase, RHA = α-L-rhamnosidase, BGL = β-1,4-D-glucosidase activity. Letters (A-F) are shown to explain the statistical differences between samples within each specific enzyme assay. Samples annotated with different letters show significant differences among the strains, while samples sharing the same letters show no statistically significant differences (ANOVA and Tukey's HDS test, $p < 0.05$).

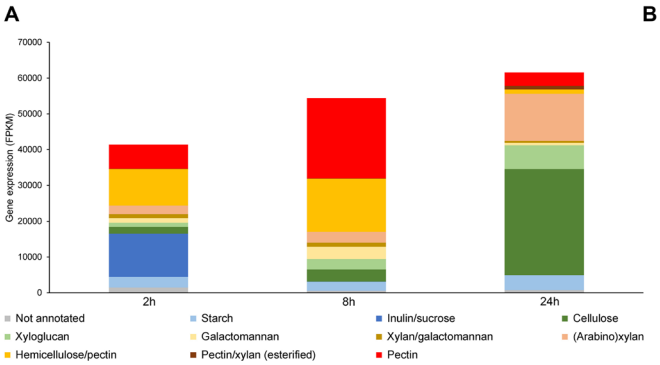
The deletion of *xlnR* and *clrB* significantly reduced endoxylanase (XLN) and endoglucanase (EGL) activities, which was also observed for Δ*xlnR*Δ*clrB*. Moreover, the deletion of *clrB* had a higher impact on the endogalactanase (GAL), arabinofuranosidase (ABF) and β-galactosidase (LAC) activities than that of *xlnR*. These activities contribute to the degradation of pectin, which is abundantly present in sugar beet pulp. However, the growth profile results do not indicate a major involvement of ClrB in the degradation of sugar beet pectin (Figure 1). Deletion of *rhaR* or *galX* resulted in overall little or no change in the measured activities compared to the reference strain. In contrast, the deletion of *araR* or *gaaR* abolished GAL activity, while the Δ*araR* strain also showed abolished ABF activity. All combinatorial deletion mutants carrying the deletion of *araR* (Δ*xlnR*Δ*araR*, Δ*xlnR*Δ*araR*Δ*clrB*, Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* and Δ*gaaR*Δ*araR* Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB*) showed abolished ABF and GAL activities, as well as highly reduced XLN and EGL activities. Interestingly, these strains also showed significantly increased α-galactosidase (AGL), LAC, α-rhamnosidase (RHA) and β-glucosidase (BGL) activities. These activities were minimal in the exoproteome of the reference, Δ*xlnR*Δ*clrB* and the single deletion strains. These results may indicate a shift toward the utilization of alternative components of sugar beet pulp by the combinatorial deletion mutants carrying the deletion of *araR*.

2.3 Gene expression levels show the preferential use of sugar beet pulp components by *A. niger*

Transcriptome analysis of 2, 8, and 24 h liquid culture samples has been performed to assess the genetic response of *A. niger* toward the utilization of sugar beet pulp. The expression profile of CAZy-encoding genes of this fungus showed a significant change over the time course, indicating the preferential use of sugar beet pulp components at different timepoints and the adaptation of *A. niger* to the remaining carbon sources over time (Figure 3).

The exo-inulinase-encoding gene *inuE* showed the highest expression level after 2 h (Data S2), most likely as a response to the presence of sucrose, which may still be present in washed sugar beet pulp. Additionally, *sucA*, encoding an extracellular invertase, was among the highest expressed CAZy genes at this timepoint. The high expression level of these two enzymes at this early timepoint, as well as the minimal/abolished expression after 8 h and 24 h of growth indicates that *A. niger* first utilizes sucrose as a mean of quick recovery after the transfer to sugar beet pulp. Several

pectinolytic and/or hemicellulolytic genes (eg., *abfB*, *abnA*, *abfC*, *axhA*, NRRL3_8701 (putative exo-galactanase encoding gene), *gbgA*, *lacA*, *abfA*, *lacB*, *eglA*, *galA*) also showed high expression levels (FPKM > 500) in the reference strain after 2 h of growth.



B

Substrate	2h	8h	24h
Pectin	22	34	30
Pectin/xylan (esterified)	1	2	3
Hemicellulose/pectin	9	11	9
(Arabino)xylan	6	7	8
Xylan/galactomannan	5	5	4
Galactomannan	3	3	4
Xyloglucan	5	8	8
Cellulose	9	13	19
Inulin/sucrose	3	1	0
Starch	10	10	12
Not annotated	11	8	9

Number of genes
Low
High

Figure 3. Expression of CAZy-encoding genes in the reference strain (CBS 138852). A) Cumulative expression of genes associated with the degradation of specific substrates after 2 h, 8 h and 24 h of growth in 1% sugar beet pulp liquid cultures. Substrates are shown in the figure legend. **B**) Number of genes associated with the degradation of each substrate at individual timepoints. Only genes with an expression value of FPKM > 20 were considered for this analysis.

Expression analysis of genes involved in the primary sugar metabolic pathways (D-galacturonic acid pathway (Figure 4A); L-rhamnose pathway (Figure 4B); D-galactose pathways (Figure 4C); pentose catabolic pathway (PCP) (Figure 4D) and glycolysis (Figure 4E)) showed that the expression of the PCP genes was the highest at this stage of growth (Figure 4D). This result correlates with the expected high release of L-arabinose catalyzed by the aforementioned CAZymes.

The relative expression of pectinolytic and hemicellulolytic (including (arabino)xylanases, galactomannanases, xyloglucanases) genes was higher after 8 h (Figure 3). The genes encoding pectinolytic enzymes showed the highest increment in expression (Data S2), indicating that *A. niger* mainly utilizes pectin at this timepoint. This is further supported by the increased expression of genes involved in D-galacturonic acid metabolism (*gaaA, gaaB*, *gaaC* and *larA*) (Figure 4A), which is the main constituent of the pectin backbone.

The expression level of cellulolytic genes was relatively low after 2 h or 8 h, which correlates with our observation that cellulose is utilized only at a later stage of growth (Figure 1). However, the significant decrease in the expression of pectinolytic genes, as well as the increase in case of cellulolytic genes (eg., *cbhB*, *cbhC*, *eglA*, *bgl4*, *bglA*, *eglC*, *bglM*, NRRL3_3383 (putative lytic polysaccharide monooxygenase (LPMO)) and

NRRL3_6436 (putative β-glucosidase) (Data S2) after 24 h, indicates a shift toward cellulose utilization at this timepoint (Figure 3).

Several genes coding for enzymes involved in starch degradation (eg., *agdB*, *glaA*, *amyA*, *agdA*, *aamA*) (Data S2) also showed an overall consistent expression at each timepoint. However, considering the composition of sugar beet pulp, it was not likely that the fungus utilized starch for growth at any of the studied timepoints. Most likely the presence of low levels of D-glucose in the medium induced the production of the aforementioned starch-degrading enzymes mediated by the amylolytic regulator AmyR[27,28].

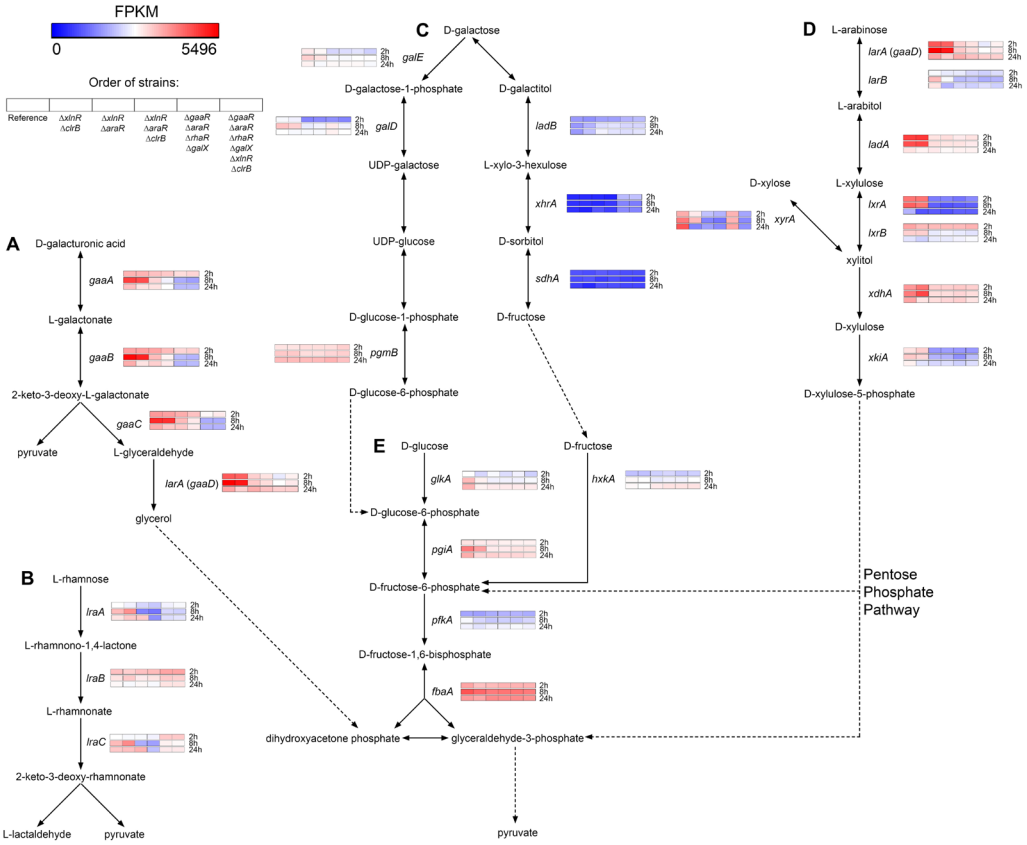


Figure 4. Hierarchical clustering of genes involved in the major primary carbon metabolic pathways in the *A. niger* reference (CBS 138852) and selected combinatorial deletion strains. Gene expression data are presented for the D-galacturonic acid pathway (**A**), L-rhamnose pathway (**B**), the Leloir and oxidoreductive D-galactose pathway (**C**), Pentose Catabolic Pathway (**D**) and glycolysis (**E**). Dashed lines indicate the connections between different pathways when applicable.

2.4 The contribution of major TFs toward the degradation of sugar beet pulp is time dependent

Among the strains of this study, only the strains carrying the deletion of *araR* (Δ*araR,* Δ*xlnR*Δ*araR*, Δ*xlnR*Δ*araR*Δ*clrB*, Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*, Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB*) showed a different CAZyme pattern than the reference strain after 2 h of growth (Figure S2, clusters F and G). However, the reduced or impaired pectinolytic and hemicellulolytic activities in these deletion mutants did not result in the upregulation of genes involved in the degradation of other components, such as cellulose, at this timepoint. Additionally, none of the single or combinatorial TF deletions resulted in a significant change (fold change > 2 or < 0.5, and *p*adj < 0.01) in the expression level of *inuE* and *sucA* (Figure S2). These results support the primary utilization of sucrose by each deletion mutant after 2 h of growth.

In contrast, after 8 h and 24 h, several single and combinatorial deletion mutants indicated substantial alteration in the utilization of sugar beet pulp components. Hierarchical clustering of the expression of CAZy-encoding genes after 8 h highlighted the important role of AraR and GaaR in the degradation of sugar beet pulp (Figure 5). Both single deletions resulted in the downregulation of a wide range of pectinolytic genes (Figure 5, cluster G). Moreover, the deletion of *araR* resulted in the downregulation of additional genes encoding accessory enzymes required for the efficient degradation of pectin (*gbgA*, *abfB*, *lacA*) (Figure 5, clusters H and I). The gene encoding the repressor of D-galacturonic acid utilization, GaaX[29], showed high expression in the reference strain after 8 h of growth, when the fungus was most likely utilizing pectin as primary carbon source. Both the single and combinatorial *araR* and *gaaR* deletion mutants showed the downregulation of *gaaX* after 8 h compared to the reference strain (Figure 6). However, only the mutants carrying the deletion of *gaaR* showed downregulation of *gaaX* after 24 h of growth (Figure 6).

Several genes were also upregulated in Δ*araR* and/or Δ*gaaR*. Several cellulolytic genes (eg., *cbhB*, *eglA*, *eglC*, and NRRL3_6436 (putative β-glucosidase encoding gene)) (Figure 5, cluster K) were highly expressed when either *araR* or *gaaR* was deleted. Moreover, the β-glucosidase encoding genes *bglM* and *bgl4*, the cellobiohydrolase encoding genes *cbhA* and *cbhC*, as well as the putative LPMO encoding gene NRRL3_3383 were also upregulated in the *araR* deletion mutant (Figure 5, clusters J and K). The majority of these cellulolytic genes (*cbhB*, *cbhC*, *cbhD*, *eglA*, NRRL3_3383) showed minimal expression in the reference strain, which correlates with the increased number of cellulolytic genes expressed in the Δ*araR* and Δ*gaaR* mutants (Figure S3).

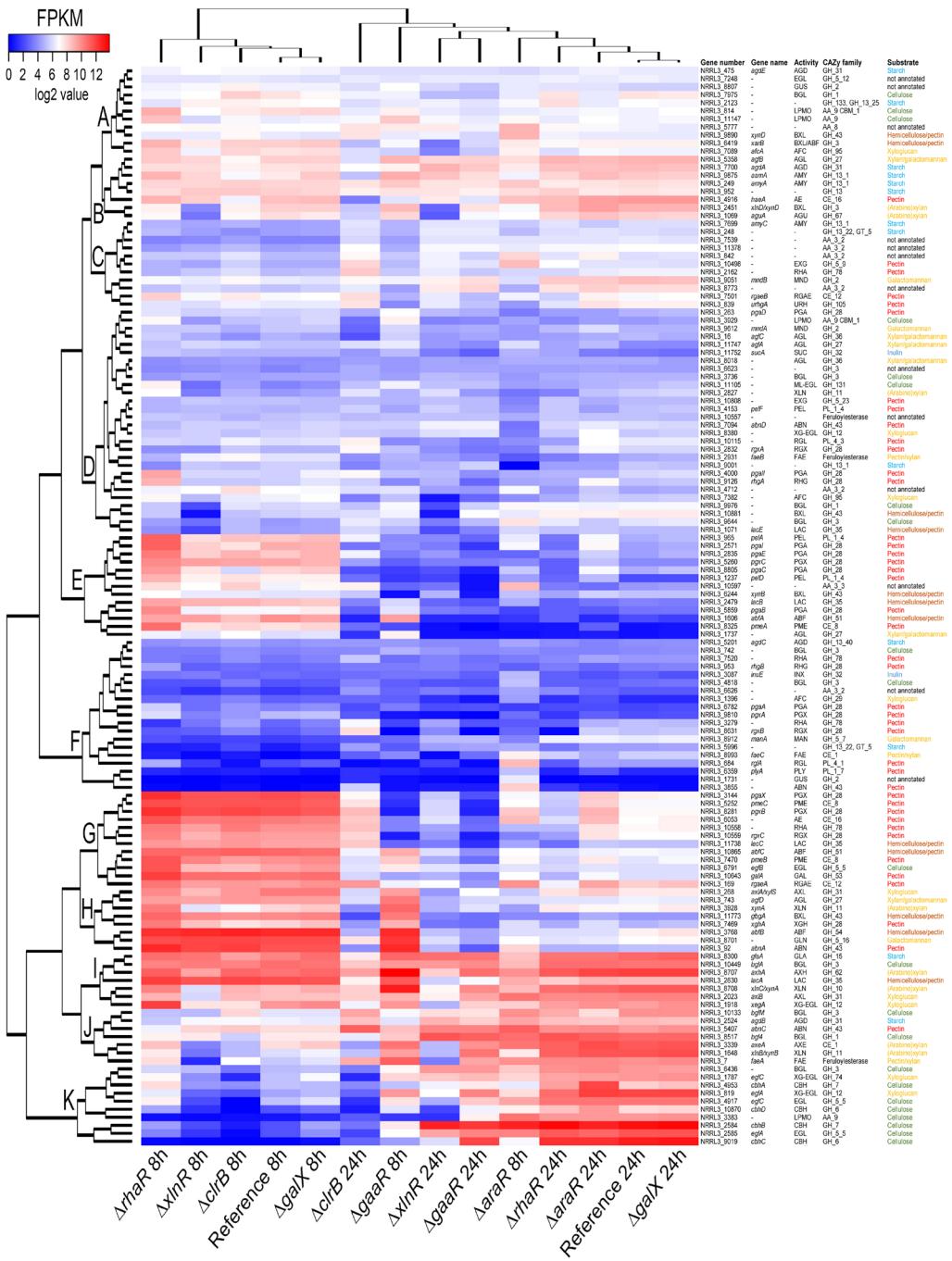


Figure 5. Hierarchical clustering of CAZy-encoding genes in the *A. niger* reference (CBS 138852) and single deletion mutants. Gene expression data originated from 8 to 24 h of growth in 1% sugar beet pulp liquid cultures. The substrates associated with the corresponding genes are indicated by different colors. Enzyme activity abbreviations are described in Table S2.

Moreover, the increased cellulolytic gene expression also correlates with the higher expression of *clrB* in Δ*araR* (Figure 6). These results suggest an early switch toward the utilization of cellulose in the studied deletion mutants. Moreover, a possible shift toward the utilization of hemicellulose components has been observed in these two single deletion mutants. Hemicellulose-specific upregulated genes include *axeA*, *xlnB/xynB*, *faeA*, *eglA* and *eglC* (Figure 5, clusters J and K). Overall, the deletion of *gaaR* further increased the expression of these genes compared to the deletion of *araR*, and resulted in the upregulation of additional (arabino)xylanase encoding genes, such as *xynA*, *axhA* and *xlnC*/*xynA* (Figure 5, clusters H and I; Figure S3).

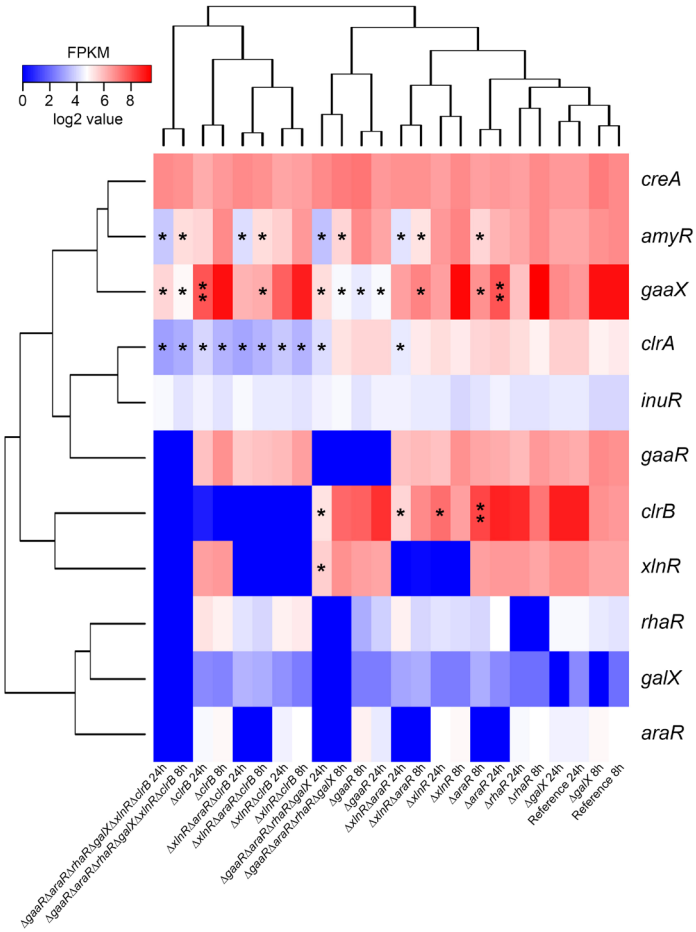


Figure 6. Hierarchical clustering of transcription factor genes in the *A. niger* reference (CBS 138852) and deletion mutant strains. Gene expression data originated from 8 to 24 h of growth in 1% sugar beet pulp liquid cultures. Genes that are downregulated compared with the reference (fold change < 0.5; *p*adj < 0.01) are indicated by an asterisk (*). Genes that are upregulated compared with the reference (fold change > 2; *p*adj < 0.01) are indicated by two asterisks (**).

After 24 h, the reference strain showed the high expression of several (hemi-)cellulases (Figure 5, clusters J and K), indicating mainly the utilization of cellulose and xyloglucan at this stage of growth. Based on the hierarchical clustering of CAZy-encoding genes, the *xlnR* and *clrB* single deletion mutants showed a distinct pattern from that of the reference strain. In Δ*xlnR*, a broad range of CAZy genes encoding several (hemi-) cellulases (Figure 5, clusters H-K), as well as several pectinolytic enzymes, such as *pgaX*, *pmeC* or *pgxB* (Figure 5, cluster G) were downregulated. Although the deletion of *xlnR* did not result in the upregulation of any CAZy-encoding genes, several cellulolytic genes, such as *cbhB* and *bgl4* were the most highly expressed CAZyme genes in this mutant (Figure 5, clusters J and K; Figure S4). This result may suggest an attempt of the fungus to use the cellulose present in sugar beet pulp, although the growth profile showed the inability of Δ*xlnR* to grow on this component (Figure 1).

In contrast, the deletion of *clrB* resulted in both the downregulation and upregulation of several CAZy-encoding genes, mainly affecting cellulose utilization (Figure 5, clusters J and K). Moreover, the expression of the cellulolytic regulator encoding gene *clrA* was reduced in the *clrB* deletion mutant (Figure 6), which may explain (part of) the reduction of the expression of cellulolytic genes. Additionally, a large number of hemicellulolytic genes were downregulated in this mutant (Figure 5, clusters H-K), mainly affecting the utilization of xyloglucan. However, several genes involved in pectin utilization were upregulated compared to the reference strain (Figure 5, cluster G; Figure S4), indicating that in contrast to the reference strain, the *clrB* deletion mutant did not shift to the utilization of cellulose after 24 h, and it continuously utilized the residual pectin found in sugar beet pulp for growth.

Based on the hierarchical clustering of CAZyme genes at both 8 and 24 h, the deletion of *galX* did not result in differential expression of any of the genes analyzed.

2.5 Combining TF deletions forces *A. niger* to switch to the utilization of alternative carbon sources

Transcriptome analysis of combinatorial deletion mutants was assessed to evaluate the interactions between TFs within the regulatory network governing sugar beet pulp degradation. The Δ*xlnR*Δ*clrB* double mutant was primarily expected to have an impact on cellulose and xyloglucan utilization. This mutant did not show a distinct CAZyme profile from that of the reference strain after 8 h, as it did not have a significant impact on the pectinolytic system. Thus, its CAZyme gene expression profile mostly resembles

that of the reference strain. However, after 24 h, the Δ*xlnR*Δ*clrB* mutant showed a strong downregulation of major (hemi-)cellulolytic genes (Figure 7, see clusters B, C and F as examples). Most pectinolytic genes were not affected in this mutant, suggesting that it mainly utilizes residual pectin for growth, similar to Δ*clrB* after 24 h (Figure S4).

All combinatorial deletion mutants except for Δ*xlnR*Δ*clrB* showed a strong downregulation of the major pectinolytic genes (Figure 7, clusters D, G, H and I) after 8 h. However, several (putative) pectinolytic genes (*abnC*, NRRL3_10498 (putative exo-galactanase encoding gene), and NRRL3_3855 (putative endo-arabinanase encoding gene)) (Figure 7, cluster J) were highly upregulated in most combinatorial deletion strains after both 8 h and 24 h. While most combinatorial deletion mutants showed the upregulation of these pectinolytic genes, the quadruple deletion strain Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* also showed the upregulation of several cellulolytic genes (*bglM*, *blg4*, NRRL3_9644 (putative β-glucosidase encoding gene), NRRL3_3383 (putative LPMO encoding gene), *cbhB*, and *eglA*) (Figure 7, clusters B, E and F). All these genes except for *bglM* and *bgl4*, showed minimal to no expression in the reference strain, indicating that the quadruple deletion mutant may have switched to the utilization of cellulose after 8 h of growth, similar to Δ*araR* and Δ*gaaR* (Figure 5, Figure S3).

Moreover, all strains, including the reference, showed expression of proteolytic genes after 8 h of growth (Figure S5), which was higher in mutants carrying the *araR* deletion. The aspartic peptidase gene *pepA* showed the highest increase in expression in the *araR* deletion mutants. The expression of proteolytic genes was, in general, lower at 24 h, with the exception of Δ*xlnR*Δ*clrB*, most likely as an alternative for the inability to utilize cellulose at this timepoint. In contrast, the Δ*xlnR*Δ*araR*Δ*clrB* mutant showed a low expression of selected proteolytic genes (Figure S5), as well as an overall strongly downregulated CAZyme gene profile comparable to that of the Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* mutant (Figure 7). This indicates that both strains are most likely unable to efficiently utilize any of the sugar beet pulp components after 24 h of growth.

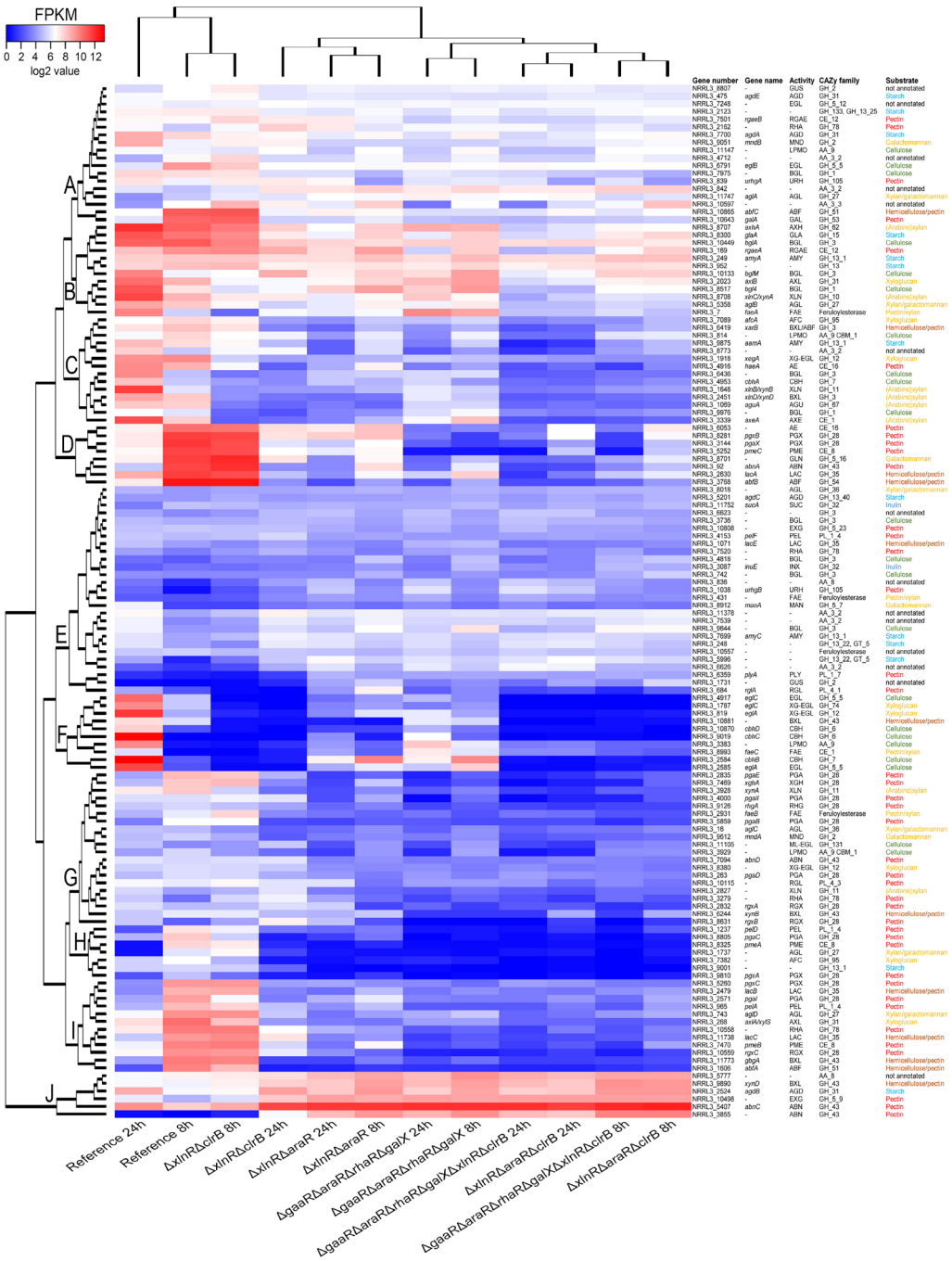


Figure 7. Hierarchical clustering of CAZy-encoding genes in the *A. niger* reference (CBS 138852) and combinatorial deletion mutants. Gene expression data originated from 8 to 24 h of growth in 1% sugar beet pulp liquid cultures. The substrates associated with the corresponding genes are indicated by different colors. Enzyme activity abbreviations are described in Table S2.

3. Discussion

In the bio-based economy, it is crucial to understand the fungal regulatory network governing plant biomass degradation to facilitate the generation of fungal strains with improved abilities to degrade major plant biomass substrates. In this study, we assessed the regulatory network of *A. niger* involved in the degradation of a bulk agro-industrial waste material, sugar beet pulp. We generated a broad set of single and combinatorial deletion mutants targeting the pectinolytic regulators GaaR, AraR, RhaR and GalX as well as the (hemi-)cellulolytic regulators XlnR and ClrB to evaluate the degradation of major sugar beet pulp components.

Sugar beet pulp mainly consists of pectin, cellulose and xyloglucan[5], as confirmed by the sugar composition analysis (Table S1). The process of sugar beet pulp degradation by *A. niger* in both solid and liquid cultures has already been reported[30,31]. Moreover, the contribution of three major TFs to the degradation of sugar beet pectin was previously assessed[20]. However, the contribution of the major TFs to the overall degradation of sugar beet pulp by *A. niger* has not been reported in detail. Transcriptome data of the *A. niger* reference (CBS 138852) strain showed the preferential use of sugar beet pulp components of this fungus, which correlates with previously reported observations[31]. Our data confirmed that after 2 h, sucrose was the primary carbohydrate of sugar beet pulp that was utilized by *A. niger*. The gene showing the overall highest expression level in all strains at this timepoint was *inuE*, encoding an exo-inulinase, which has been reported to be highly expressed in the presence of sucrose[32]. However, the expression of metabolic genes also suggests an early utilization of L-arabinose residues, indicated by the expression of major PCP genes involved in its metabolism. Thus, the response to the presence of sucrose is closely followed by the degradation of pectin, which is most likely followed by the degradation of hemicellulose (e.g., xyloglucan), supported by the high expression level of genes related to degradation of these substrates after 8 h and 24 h of growth. Cellulose serves as a last resort substrate, mainly evidenced by the 24 h transcriptome data. The response to the presence of cellulose is most likely triggered when the pectin and hemicellulose content is depleting. The slow utilization of cellulose by *A. niger* was already described[15,31], and is further supported by our growth profile results, showing growth only after long incubation times.

Among all the single deletion mutants, the Δ*gaaR* strain had the strongest reduction in growth on sugar beet pectin, as previously reported[20]. However, the deletion of *clrB* resulted in the overall strongest growth defect on sugar beet pulp solid medium, considering the whole composition of this substrate. In contrast, in liquid cultures, the

deletion of *araR* had the strongest effect on the exoproteome after both 8 h and 24 h, as evidenced by SDS-PAGE analysis. The phenotypic difference between these mutants is most likely related to the different experimental time course and culturing condition, that cannot be directly compared[31].

Moreover, transcriptomic data supported the highest impact on CAZyme production by the deletion of *araR* after 2 h, as Δ*araR* showed a distinct CAZyme pattern compared to that of the reference strain after 8 h. The deletion of *gaaR* resulted in a comparable effect on CAZyme production to that of the Δ*araR* strain after 8 h, showing a higher impact than the deletion of *araR* after 24 h. These results confirm that AraR has a more important role in the initial degradation of pectin, regulating the release of L-arabinose units from the arabinan and arabinogalactan side chains of pectin, while GaaR is more involved in the degradation of the galacturonan backbone[20]. Moreover, the deletion of *araR* and *gaaR* indicated a shift toward the utilization of (hemi-)cellulose, evidenced by the increased expression of (hemi-)cellulolytic genes. The upregulation of XlnR-regulated (hemi-)cellulolytic genes has previously been suggested to occur when *gaaR* is deleted[20]. After 24 h, the deletion of *clrB* showed the highest impact on CAZyme production, highly reducing the expression of genes encoding CAZymes involved in the degradation of cellulose and xyloglucan.

Our results also show that the deletion of *galX* had only a minor phenotypic impact, evidenced by the further reduced growth of Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* compared to the Δ*gaaR*Δ*araR*Δ*rhaR* mutant on L-arabinose. Transcriptome data showed no differential expression of CAZyme or metabolic genes in the Δ*galX* mutant compared to the reference strain at any of the studied timepoints. These results suggest that GalX is not a main TF involved in sugar beet pulp degradation, but it contributes to the utilization of L-arabinose and D-galactose[22]. ARA1, the functional ortholog of AraR in *Trichoderma reesei*, has been reported to regulate D-galactose catabolism in this fungus[33]. However, no correlation between D-galactose responsive regulators and L-arabinose utilization has been reported yet. In *Aspergillus nidulans,* GalX was shown to control GalR, another D-galactose-responsive regulator only present in this species[34], but is not involved in regulation of pentose catabolism[35]. Whether GalX would contribute to L-arabinose metabolism together with AraR in *A. niger* remains to be addressed.

Our results show that the abolished growth on a major component does not indicate the highest impact on the growth on a complex substrate such as sugar beet pulp. Owing to the complexity and redundancy of the regulatory network involved in plant biomass degradation, which is well indicated by the co-regulation of several crucial genes[15,20,24,36],

the analysis of combinatorial deletion strains is required to better understand the regulation of the degradation of specific substrate components.

As expected, the double deletion of *xlnR* and *araR* had a high impact on sugar metabolism, as evidenced by the downregulation of major PCP genes as well as the reduced growth on D-xylose and L-arabinose[25]. The double deletion of *xlnR* and *clrB* resulted in the lowest impact on the expression of CAZyme genes after 8 h, and showed an overall minor reduction in the expression of metabolic genes at all analyzed timepoints. However, both XlnR and ClrB were reported to play an important role in (hemi-)cellulose utilization[15,23,26,37], which correlates with the minor phenotypic impact after 8 h, when pectin is the primary carbon source in sugar beet pulp. In contrast, the Δ*xlnR*Δ*clrB* mutant showed a more distinct phenotype after 24 h when the utilization of cellulose was more prominent in the reference strain. The CAZyme gene expression profile of Δ*xlnR*Δ*clrB* mutant indicated that most likely pectin, which was still present in the medium, was the primary carbohydrate utilized by this mutant after 24 h of growth. Moreover, the expression of proteolytic genes was the highest in the Δ*xlnR*Δ*clrB* after 24 h, which supports a reduced ability to grow by utilizing carbohydrates at this timepoint.

The significant increase in expression of the putative pectinolytic genes *abnC*, NRRL3_10498 (putative exo-galactanase) and NRRL3_3855 (putative endo-arabinanase) in most combinatorial deletion mutants indicates that these genes might be part of back-up system when the expression of major pectinolytic genes is reduced. This hypothesis is also supported by the fact that only the Δ*xlnR*Δ*clrB* double deletion mutant, which still shows expression levels of most major pectinolytic genes comparable to the reference strain, did not show a substantial increase in the expression of these putative back-up genes after 8 h.

Interestingly, all tested strains showed the expression of several genes encoding amylolytic proteins (e.g., *agdB*, *glaA*, *amyA*, *agdA*) in our experiment, despite the absence of starch in sugar beet pulp. These genes are not likely to be directly affected by the studied TFs, and the expression through the amylolytic regulator, AmyR, might be a result of the presence of the released D-glucose in the medium[28].

Overall, this study shows that the single and combinatorial deletion of *araR* resulted in a highly altered phenotype, supported by a distinct CAZyme gene expression profile after 2 h and 8 h of growth, indicating a shift toward the utilization of alternative carbon sources at the later timepoint. Moreover, the transcriptome data of 8 h samples suggested the additional utilization of proteins in the combinatorial deletion strains carrying the deletion of *araR*, evidenced by the increased expression of proteolytic genes, such as *pepA* or

NRRL3_800 (putative tripeptidyl peptidase). The enzyme activity studies also supported a distinct approach for the utilization of sugar beet pulp components for these mutants.

Finally, the Δ*xlnR*Δ*araR*Δ*clrB* and the Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* mutants showed comparable CAZyme gene expression profiles at all timepoints, as well as comparable proteolytic gene expression profile at the analyzed 8 h and 24 h. The data suggests that both of these strains are likely starving after 24 h in the sugar beet pulp liquid cultures. Even though the Δ*xlnR*Δ*araR*Δ*clrB* mutant showed a substantially improved growth compared to the Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* mutant on solid medium, the overall data indicate that AraR, XlnR and ClrB are responsible for the regulation of the major activities involved in the efficient degradation and utilization of sugar beet pulp components.

4. Limitations of the study

The current study shows phenotypic characterization of single and combinatorial deletion mutants of the main TFs involved in sugar beet pulp utilization. Moreover, SDS-PAGE, enzymatic assays and gene expression profiles of selected strains have been shown, compared and discussed. Whereas the number of genetic modifications that we can perform in *A. niger* with CRISPR/Cas9 is not a limiting factor, owing to the very high number of strains generated with the combinatorial gene deletion approach, it is not possible to analyze and show all of them in this study. Thus, only a selection of strains was analyzed. The transcriptome data generated in this study shows the adaptation of *A. niger* toward the utilization of alternative components of sugar beet pulp when several TFs are deleted. These data also suggest the upregulation of putative pectinolytic genes. Proteomics would also help validate the gene expression data showed in this study through the analysis of the presence of the corresponding proteins in the exoproteome. However, proteomics analyses have not been performed in this study.

5. STAR Methods

5.1 Resource availability

5.1.1 Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ronald P. de Vries (r.devries@wi.knaw.nl).

5.1.2 Materials availability

All fungal strains generated in this study were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) under the accession numbers listed in Table S3.

5.1.3 Data and code availability

The RNA-seq data generated in this study have been deposited at the Sequence Read Archive at NCBI (Accession numbers: SRP296371 - SRP296379, SRP296381 - SRP296396, SRP296398 - SRP296424, SRP296426 - SRP296434, SRP296436 - SRP296444, SRP299075 - SRP299083, SRP299091 - SRP299099, SRP308099 - SRP308107, SRP332363 - SRP332366 and SRP332368 - SRP332371).

All other data are available in the main text or in the supplemental information files.

5.2 Experimental model and subject details

5.2.1 Microbial strains and growth conditions

Escherichia coli DH5α was used for plasmid propagation and was grown in Luria-Bertani (LB) medium with 50 µg/mL ampicillin (Sigma-Aldrich) at 37°C.

All fungal strains used in this study (Table S3) are derived from *A. niger* CBS 138852 and were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). They were grown and maintained at 30ºC on *Aspergillus* Complete Medium (CM)[38] containing 1% D-glucose and supplemented with 1.22 g/L

uridine (Sigma-Aldrich). For conidia collection, spores were harvested, dispersed in ACES buffer, and concentration was adjusted using a hemocytometer.

Growth profiles were performed on Minimal Medium (MM)[38] plates containing 25 mM D-glucose, L-arabinose, D-xylose, D-galacturonic acid or L-rhamnose (Sigma-Aldrich); or 1% beechwood xylan, cellulose, xyloglucan, sugar beet pectin or sugar beet pulp. Total sugar composition of the sugar beet pulp used in this study is shown in Table S1. In order to remove residual free sugars, dried and finely ground sugar beet pulp was autoclaved and washed as previously described[15]. All media were supplemented with 1.22 g/L uridine. Growth profile plates were inoculated in duplicates with 10^3 conidia and incubated at 30°C for up to 14 days. Growth was monitored daily by visual inspection.

5.3 Method details

5.3.1 DNA constructions and fungal transformation

All strains generated in this study were obtained using the CRISPR/Cas9 genome editing system[39]. The autonomous replicative plasmid ANEp8-Cas9-*pyrG* was used in this work. The *A. niger* regulators deleted in this study were AraR (gene ID: NRRL3_07564), XlnR (NRRL3_04034), ClrB (NRRL3_09050), GaaR (NRRL3_08195), RhaR (NRRL3_01496), and GalX (NRRL3_07290). The design of the gRNA sequences (20 bp) was performed using the Geneious 11.04.4 software tool (https://www.geneious.com). The gRNA sequences (Table S4) with no predicted off-targets and the highest on-target activity were designed based on the experimentally determined predictive model described by Doench et al.[40]. All rescue templates (RTs), which include ~500 bp of the 5' and 3' flanking regions of the target genes, were obtained by fusion-PCR using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Two PCR fragments were generated by amplifying ~600 bp upstream and downstream sequence of the target genes. These two fragments were fused together in a second nested PCR obtaining ~1,000 bp RT, and were subsequently purified (Wizard® SV Gel and PCR Clean-Up System, Promega).

CRISPR/Cas9 plasmid construction was performed following previously described protocols[39,41]. The generation and transformation of *A. niger* protoplasts were carried out as previously reported[20] with some modifications[41]. One µg ANEp8-Cas9-*pyrG* plasmid, together with 5 µg of each corresponding RT were used for each transformation. Putative mutant strains were purified by two consecutive single colony streaking, followed by

cultivation on uridine-containing plates in order to remove the self-replicating plasmid. Candidates carrying the expected mutations were subsequently grown on medium containing 5-fluoro-orotic acid (5-FOA) in order to screen for colonies which have lost the ANEp8-Cas9-*pyrG* plasmid. Single Δ*xlnR*, Δ*araR,* Δ*clrB,* Δ*gaaR,* Δ*rhaR* and Δ*galX* mutants were obtained by single deletion using *A. niger* CBS 138852 as genetic background. The Δ*xlnR*Δ*araR* and Δ*xlnR*Δ*clrB* strains were obtained by simultaneous double deletions using the same parental strain as a background. The triple mutants Δ*xlnR*Δ*araR*Δ*clrB* and Δ*gaaR*Δ*araR*Δ*rhaR* were obtained after a single *araR* deletion in the Δ*xlnR*Δ*clrB*, and Δ*gaaR*Δ*rhaR* genetic backgrounds, respectively. The quadruple Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* was obtained after simultaneous *araR* and *galX* deletions in the Δ*gaaR*Δ*rhaR* genetic background. The quintuple Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR* and Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*clrB* mutant strains were obtained after single *xlnR* or *clrB* deletions in the Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* genetic background, respectively. Finally, the sextuple mutant Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* was obtained after a simultaneous double deletion of *xlnR* and *clrB* in Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* genetic background. Mutant strains were confirmed phenotypically (Figure 1) and by analytical PCR through the amplification of each target gene region (data not shown). All primers used in this study are shown in Table S4 and were ordered from Integrated DNA Technologies, Inc. (IDT, Leuven, Belgium).

5.3.2 Protein production and enzyme activity assays

Cell free supernatants of 1% washed sugar beet pulp samples from *A. niger* reference strain, Δ*xlnR*, Δ*araR,* Δ*clrB,* Δ*gaaR,* Δ*rhaR,* Δ*galX,* Δ*xlnR*Δ*araR,* Δ*xlnR*Δ*clrB,* Δ*xlnR*Δ*araR*Δ*clrB,* Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* and Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* mutant strains were harvested after 2, 8 and 24 h of growth at 30ºC and 250 rpm. Ten µL of each supernatant sample were analyzed by SDS-PAGE using SDS-12% polyacrylamide gels calibrated with PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific), silver stained[42], and documented using HP Scanjet G2410 scanner. Samples were evaluated in biological duplicates.

Enzyme activities were evaluated by using colorimetric *para*-nitrophenol (*p*NP) or azo-dye substrate assays in 96-well flat bottom microtiter plates. For *p*NP assays, 10 µL 24 h supernatant samples were mixed with 50 µL of 50 mM NaAc (pH 5), 30 µL of demineralized water and 10 µL of 0.1% 4-nitrophenyl α-L-arabinofuranoside (for α -arabinofuranosidase (ABF) activity), 0.1% 4-nitrophenyl α-D-galactopyranoside (for α-galactosidase (AGL) activity), 0.1% 4-nitrophenyl β-D-galactopyranoside (for β-galactosidase (LAC) activity),

0.1% 4-nitrophenyl α-L-rhamnopyranoside (for α-rhamnosidase (RHA) activity) or 0.1% 4-nitrophenyl β-D-glucopyranoside (for β-glucosidase (BGL) activity) in a final volume of 100 µL. The assays were measured after 30-360 min incubation at 30°C. The reactions were stopped by adding 100 µL of 0.25 M Na_2CO_3 to the mixture and the absorption values were measured at 405 nm wavelength using a FLUOstar OPTIMA microplate reader (BMG Labtech).

For azo-dye substrate assays, 20 µL 24 h supernatant samples were mixed with 30 µL of 100 mM NaAc (pH 4.6) and 50 µL of Azo-galactan (potato) (Megazyme) (for β-galactanase (GAL) activity), Azo-Xylan (Birchwood) (Megazyme) (for endoxylanase (XLN) activity) or Azo-CM-Cellulose (Megazyme) (for endoglucanase (EGL) activity). The reaction mixtures were incubated for 4 h at 30°C and were terminated by the addition of 250 µL of precipitation solution (4% NaAc*3H_2O, 0.4% ZnAc, 76% EtOH, pH 5). The microtiter plates were centrifuged at 4°C, 1000 × *g* for 10 min. Subsequently, the supernatant samples were transferred to another microtiter plates and the activity was determined based on the absorption measured at 600 nm wavelength using a FLUOstar OPTIMA microplate reader (BMG Labtech).

5.3.3 Transcriptomic analysis

For transcriptomic analysis, freshly harvested spores from *A. niger* parental strain, Δ*xlnR*, Δ*araR,* Δ*clrB,* Δ*gaaR,* Δ*rhaR,* Δ*galX,* Δ*xlnR*Δ*araR,* Δ*xlnR*Δ*clrB,* Δ*xlnR*Δ*araR*Δ*clrB,* Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* and Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* mutant strains were pre-grown (10^6 spores/mL) in 250 mL 2% D-fructose CM supplemented with 1.22 g/L uridine for 16 h at 30°C in a rotary shaker at 250 rpm. After that, mycelia were harvested by filtration through sterile cheesecloth, rinsed with MM, and ~2.5 g (wet weight) mycelium was transferred into 50 mL MM containing 1% washed sugar beet pulp with 1.22 g/L uridine. Mycelia were collected after 2, 8, and 24 h, frozen in liquid nitrogen, and stored 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 was extracted from grinded mycelia using TRIzol reagent (Invitrogen) and purified with NucleoSpin RNA kit for RNA purification (Macherey-Nagel) with DNAse treatment. RNA quality and quantity were assessed by RNA gel electrophoresis and NanoDrop ND-1000 (Thermo Scientific). Purification of mRNA, synthesis of cDNA library and sequencing were conducted at Joint Genome Institute (JGI, California, US). RNA samples were single-end sequenced using Illumina NovaSeq platform (http://illumina.com). Raw fastq file reads were filtered and trimmed using the JGI quality

control (QC) pipeline. Using BBDuk (https://sourceforge. net/projects/bbmap/) raw reads were evaluated for artefact sequence by kmer matching (kmer = 25), allowing one mismatch and detected artefact was trimmed from the 3′ end of the reads. RNA spike-in reads, PhiX reads and reads containing any undetermined nucleotides (Ns) were removed. Quality trimming was performed using the phred trimming method set at Q6. Reads under the length threshold were removed (minimum length 25 bases or 1/3 of the original read length - whichever is longer). The cleaned reads were mapped to *A. niger* NRRL3 genome (https://mycocosm.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html)[43] using HISAT2 version 2.2.0[44]. Strand-specific coverage files were generated using deepTools v3.1[45]. FeatureCounts[46] was used to generate the raw gene counts file using gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts. Raw gene counts were used to evaluate the level of correlation between biological replicates using Pearson's correlation. Three biological replicates were prepared and sequenced for each condition. Three individual samples were discarded for further analysis due to their poor sequencing quality.

Differentially expressed genes (DEGs) were detected using the R package DESeq2[47]. Transcripts were considered differentially expressed if the DESeq2 fold change of mutant strains 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 transcriptome 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 is shown in Data S2. Genes with an expression of FPKM < 20 in each sample, were excluded from the analysis.

5.4 Quantification and statistical analysis

Enzymatic activity assays were performed by using biological duplicates and technical triplicates. Differences in enzyme activities were determined using the one-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test (Table S5). Statistical significance was referred for $p < 0.05$. Analyses were done using STATGRAPHICS Centurion XVI Version 16.1.17 (www.statgraphics.com/centurion-xvi).

Author contributions

S.G. and R.S.K. performed the experiments, analyzed the data, and wrote the original manuscript. D.B., K.K., A.L., and V.N. performed the RNA sequencing and initial analysis. I.V.G. supervised the RNA sequencing. M.P. contributed to the bioinformatics analysis. R.P.dV. designed the experiments, supervised the research, and reviewed and edited the manuscript. All authors commented on and approved the submitted manuscript.

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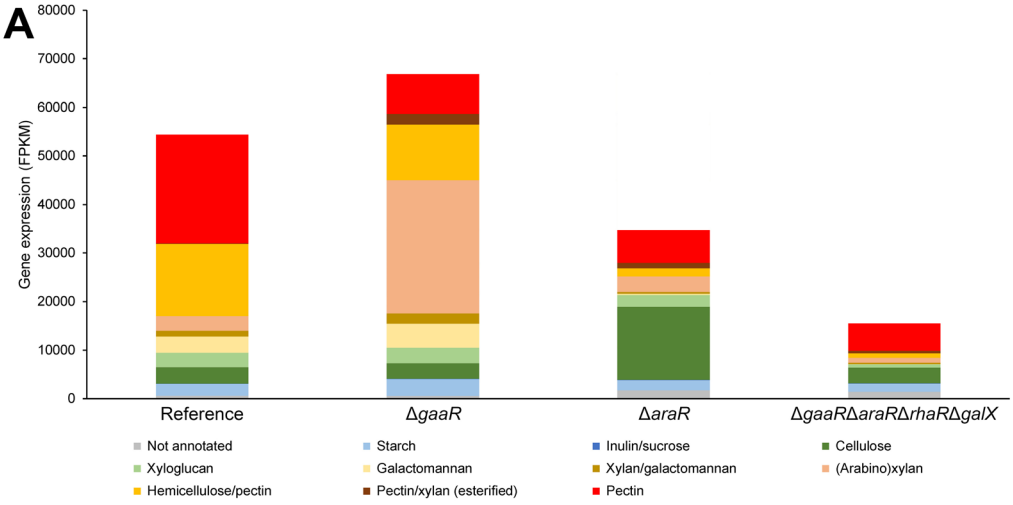
Supporting information

All supporting information are available at: https://doi.org/10.1016/j.isci.2022.104065 or can be obtained upon request from the author.

Figure S1. SDS-PAGE analysis of extracellular protein production of *A. niger* reference (CBS 138852) and regulatory mutant strains, related to Figure 2. Samples originated from 8, and 24 h growth in 1% liquid sugar beet pulp and were evaluated in biological duplicates.

Figure S2. Hierarchical clustering of CAZy-encoding genes in *A. niger* reference (CBS 138852) and deletion mutant strains, related to Figure 7. Gene expression data originated from 2 h of growth in 1% sugar beet pulp liquid cultures. The substrates associated with the corresponding genes are indicated by different colors. Enzyme activity abbreviations are described in Table S2.



B

Substrate	Reference	Δ*gaaR*	Δ*araR*	Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*
Pectin	34	17	23	10
Pectin/xylan (esterified)	2	2	2	2
Hemicellulose/pectin	11	10	10	7
(Arabino)xylan	7	8	7	6
Xylan/galactomannan	5	5	4	4
Galactomannan	3	3	2	2
Xyloglucan	8	8	7	4
Cellulose	13	18	19	14
Inulin/sucrose	1	1	2	2
Starch	10	11	12	12
Not annotated	8	7	12	10

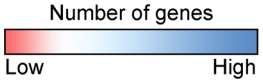
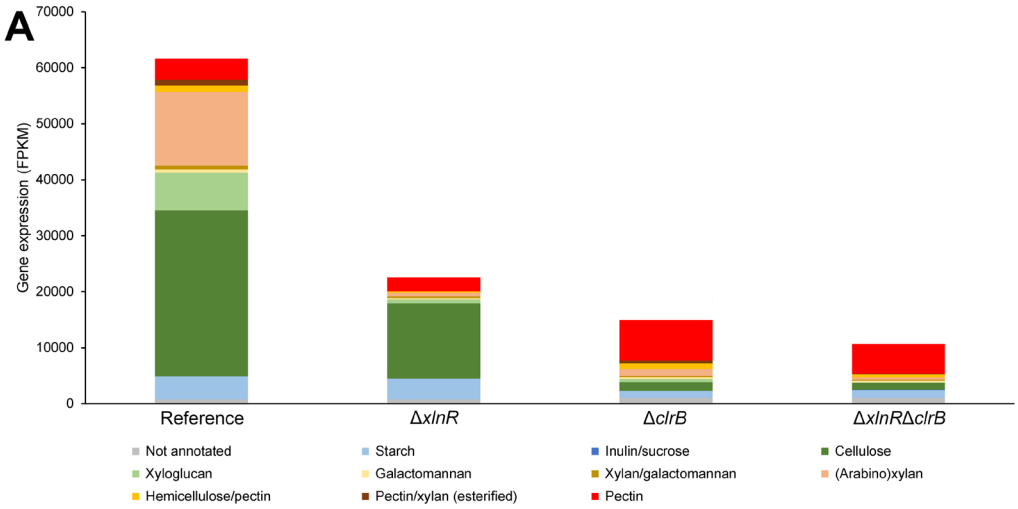


Figure S3. Expression of CAZy-encoding genes in the reference (CBS 138852) and Δ*araR*, Δ*gaaR* and Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* deletion strains, related to Figure 7. A) Cumulative expression of genes associated with the degradation of specific substrates after 8 h of growth in 1% sugar beet pulp liquid cultures. **B**) Number of genes associated with the degradation of each substrate after 8 h of growth. Only genes with an expression value of FPKM > 20 were considered for this analysis.



B

Substrate	Reference	Δ*xlnR*	Δ*clrB*	Δ*xlnR* Δ*clrB*
Pectin	30	22	28	27
Pectin/xylan (esterified)	3	0	1	1
Hemicellulose/pectin	9	5	9	5
(Arabino)xylan	8	6	6	3
Xylan/galactomannan	4	3	3	4
Galactomannan	4	4	2	2
Xyloglucan	8	5	5	2
Cellulose	19	15	11	10
Inulin/sucrose	0	0	0	1
Starch	12	11	10	11
Not annotated	9	9	11	12

Number of genes
Low
High

Figure S4. Expression of CAZy-encoding genes in the reference (CBS 138852) and Δ*clrB*, Δ*xlnR* and Δ*xlnR*Δ*clrB* deletion strains, related to Figure 7. A) Cumulative expression of genes associated with the degradation of specific substrates after 24 h of growth in 1% sugar beet pulp liquid cultures. **B**) Number of genes associated with the degradation of each substrate after 24 h of growth. Only genes with an expression value of FPKM > 20 were considered for this analysis.

Figure S5. Hierarchical clustering of genes involved in proteolytic activities in *A. niger* reference (CBS 138852) and combinatorial deletion mutant strains, related to Figure 7. The *prtT* gene encoding the proteolytic transcription factor PrtT is highlighted in bold. Gene expression data originated from 8 h and 24 h of growth in 1% sugar beet pulp liquid cultures.

Table S1. Sugar composition of sugar beet pulp used in this study, related to STAR Methods. Composition analysis was performed as previously described.

Table S2. Abbreviations of enzyme activities presented in this study, related to Figure 5 and Figure 7.

Table S3. *A. niger* strains used in this study, related to STAR Methods.

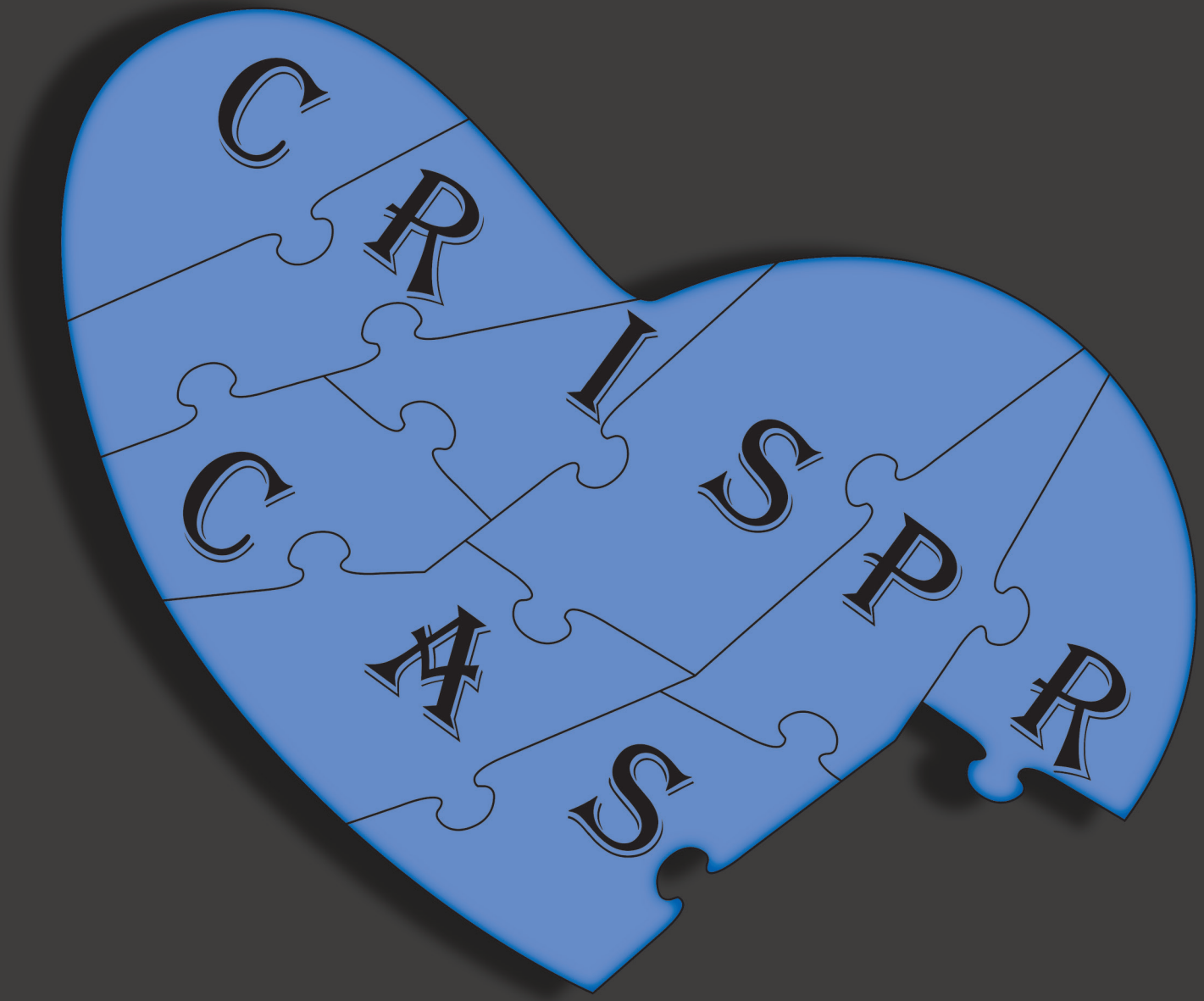
Table S4. Primers used in this study, related to STAR Methods. Homology flanks are highlighted in red.

Table S5. Summary of the ANOVA analysis for each enzymatic assay, related to STAR Methods and Figure 2.

Data S1. Enzyme activity measurements performed in this study, related to Figure 2. A) Results of *p*NP enzyme assays performed in this study. Statistical analysis was performed using the converted (nmol *p*NP/min/mL) values, while the visualization (Figure 2) was performed using the normalized mean and standard deviation (SD) values. **B**) Results of azo-substrate enzyme assays performed in this study. Statistical analysis was performed using the corrected absorbance values, while the visualization (Figure 2) was performed using the normalized mean and standard deviation (SD) values.

Data S2. Gene expression values of CAZy-, metabolic- and transcription factor genes of *A. niger* reference (CBS 138852) and mutant strains analyzed in this study, related to Figures 3-7. Samples originate from 1% sugar beet pulp liquid cultures after 2, 8 and 24 h of growth. Gene expression values of FPKM < 20 were considered low, and are indicated in red.


8


CRISPR
CAS

Chapter 9

Summary and general discussion

1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas) system is a rapidly developing molecular tool for precise genome editing. Since its establishment as a genome editing tool in 2012[1], it has been applied in a wide range of organisms, ranging from bacteria to human cell lines[2,3]. Figure 1A illustrates the number of publications reporting the application of CRISPR/Cas9 system for this purpose.

In **Chapter 2**, we summarized the applications of the CRISPR/Cas9 system in filamentous fungi in the first four years since its establishment in *Trichoderma reesei*[4] and several *Aspergillus*[5] species in 2015. However, the number of studies involving CRISPR/Cas9-mediated genome editing in filamentous fungi has rapidly increased in recent years (Figure 1B). Moreover, the establishment of CRISPR/Cas9 system in several new fungal species, such as *Penicillium subrubescens*[6], *P. digitatum*[7], *P. expansum*[7], *Fusarium proliferatum*[8] or *Ashbya gossypii*[9] was also reported. However, the increased application of CRISPR/Cas9 in filamentous fungi is partly attributed to its application in the genus *Aspergillus* (Figure 1B). In these fungi, the use of non-integrative AMA1-bearing plasmids[5,10] expressing both the *cas9* gene and the single guide RNA (sgRNA) allowed for efficient marker-free transformations[11].

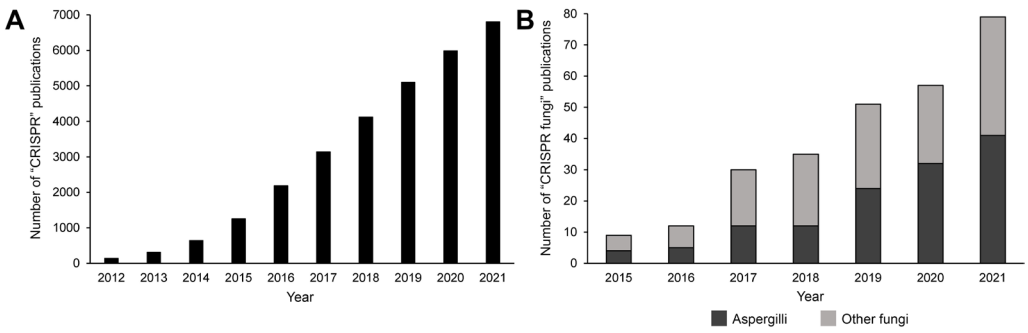


Figure 1. Number of studies associated with the CRISPR/Cas system since its establishment as a genome editing tool in 2012. Graphs represent the number of publications per year containing the terms "CRISPR" (**A**), or "CRISPR fungi" (**B**) in their titles or abstracts. Data was retrieved from PubMed in May 2022.

The CRISPR toolbox has also been expanded in filamentous fungi by the utilization of modified versions of the Cas9 protein. These involve the utilization of a nuclease-deficient Cas9 (dCas9), which was used for the activation of a silent gene cluster in *P.*

rubens[12], or the utilization of a nickase Cas9 (nCas9), used for efficient single base editing in *A. niger*[13]. Moreover, genome editing using the alternative class 2 CRISPR nuclease Cas12a/Cpf1 from *Lachnospiraceae bacterium*[14] has also been reported in *A. niger*[15] and in the thermotolerant fungi *Thermothelomyces thermophilus* and *Myceliophthora thermophila*[16]. The conventional *Streptococcus pyogenes* Cas9 (SpCas9) recognizes a 5'-NGG-3' protospacer adjacent motif (PAM), while Cpf1 recognizes a T-rich PAM sequence, 5'-TTTN-3'. This allows for targeting a larger repertoire of genomic sites with a CRISPR/Cas-system, especially AT-rich regions[14]. Additionally, the CRISPR/Cpf1 system is more suitable for multiplex genome editing than the CRISPR/Cas9 system, since Cpf1 requires only a crispr RNA (crRNA) and no trans-activating crispr RNA (tracrRNA) for this purpose[17].

With respect to commercial applications, the use of CRISPR/Cas9 or Cpf1-mediated genome editing requires royalty-bearing licenses[18]. However, an alternative to Cpf1, called MAD7 was released by Inscripta (Boulder, CO), which offers a royalty-free license for various commercial applications[19]. Recently, successful CRISPR/MAD7-mediated gene targeting was reported in *A. niger*, *A. nidulans*, *A. oryzae* and *A. aculeatus*[20], promoting new possibilities of genetic engineering in filamentous fungi.

The aim of this thesis was to apply the CRISPR/Cas9 system in *A. niger* for the generation of diverse mutations targeting transcription factors (TFs) involved in the regulation of plant biomass conversion. The generated mutations included point mutations, domain swaps and multiple subsequent gene deletions. The results of this thesis provide more insight into the possibilities of CRISPR/Cas9-mediated genome editing in *A. niger*, as well as more information about the individual roles of several TFs and their interactions within the regulatory network governing plant biomass degradation in this fungus.

2. Expanding the applications of CRISPR/Cas9 in *Aspergillus niger*

A. niger is one of the most studied filamentous fungi due to its long history of industrial applications[21]. Therefore, its ability to degrade plant biomass[22], its metabolic pathways[23,24], as well as its ability to produce primary[25] or secondary metabolites[26] has been investigated to a great extent. For this, a large number of *A. niger* mutant strains have been generated in various studies, often by performing gene deletions based on classical homologous recombination[27]. However, the number of gene deletions that can

be performed in one strain by classical gene deletion is limited by the number of available selection markers. The application of marker-free CRISPR/Cas9 transformation[11] by using a recyclable AMA1-bearing plasmid[28] can facilitate the generation of multi-deletion strains. In **Chapter 7** and **Chapter 8** we presented the generation of two large sets of TF deletion strains related to the degradation of wheat bran and sugar beet pulp, respectively.

In **Chapter 7**, a complete set of XlnR-AraR-ClrA-ClrB combinatorial deletion strains were generated in the *A. niger* N593 Δ*kusA* (Δ*ku70*) (CBS 138852) background using CRISPR/Cas9 in multiple rounds of transformations. In total 107 transformant colonies were screened, and only 2 colonies showed a comparable genotype to the parental strain, resulting in an overall 98.13% efficiency for gene deletion (Table 1). Both single and simultaneous double deletion mutants were efficiently generated, however, results showed discrepancy in the number of colonies for certain genes. The double deletion of *xlnR* and *araR* resulted in the generation of five Δ*xlnR*, two Δ*araR* and three Δ*xlnR*Δ*araR* mutants out of ten screened colonies (Table 1). A similar ratio was observed in the case of *amyR* and *inuR* double deletion, resulting in six Δ*amyR*, two Δ*inuR* and two Δ*amyR*Δ*inuR* mutants out of the ten screened colonies (**Chapter 6**). However, in case of *clrA* and *clrB* double deletion, only 8.14% (seven out of eighty-six colonies) were successful double deletion strains, likely due to the low efficiency of *clrA* deletion (Table 1). Comparable gene targeting efficiency (0-25%) was reported for CRISPR/Cas9-mediated double gene deletion in *A. niger* by using *in vitro* assembled Cas9/sgRNA ribonucleoprotein complexes[29]. However, generation of double gene knockout strains by simultaneous deletion using a dual-plasmid approach which is comparable to ours was also reported at an 89% efficiency[11]. Differences in sgRNA activity could explain the differences between the reported double gene deletion efficiencies, and further improvements of sgRNA activity prediction tools[30] could lead to more efficient generation of combinatorial deletion strains. Nonetheless, simultaneous transformations with two Cas9 plasmids harboring different sgRNA-encoding sequences and the same selection marker are an efficient method for generating combinatorial deletion mutants in *A. niger*.

Table 1. Gene deletion efficiency during the generation of *A. niger xlnR-araR-clrA-clrB* combinatorial deletion strains using CRISPR/Cas9. The number of screened colonies is indicated in bold. In case of double deletions, numbers represent the number of colonies counted for each individual single, as well as for successful double deletions.

Background strain	Single deletions		Double deletions	
	Δ*xlnR*	Δ*araR*	Δ*xlnR*/Δ*araR*/Δ*xlnR*Δ*araR*	Δ*clrA*/Δ*clrB*/Δ*clrA*Δ*clrB*
Δ*ku70*	3 out of **3**	5 out of **5**	5/2/3 out of **10**	4/7/2 out of **13**
Δ*xlnR*	-	3 out of **3**	-	5/31/2 out of **38**
Δ*araR*	-	-	-	6/13/1 out of **22**
Δ*xlnR*Δ*araR*	-	-	-	4/7/2 out of **13**

Generally, the efficiency of CRISPR/Cas9 genome editing highly depends on the used sgRNA as previously indicated, as well as on the *cas9* and sgRNA expression constructs[31]. Moreover, the transformed species or even strains[32] can influence the transformation efficiency. For instance, the first study describing the application of CRISPR/Cas9 system in another industrial *Aspergillus* species, *A. oryzae*, reported 10-20% efficiency using an integrative *cas9* gene under the control of *amyB* promoter[33]. However, using the same *cas9* expression construct in a plasmid bearing AMA1, resulted in highly improved transformation efficiency of 50-100%[34]. In this study, the improved efficiency was associated with the increased expression of *cas9* gene and sgRNA, most likely owing to the presence of multiple plasmid copies in the same cell[34]. This study further recommends the use of self-replicating plasmids for CRISPR/Cas9 genome editing, and can partly explain the high transformation efficiency observed for gene deletions in this thesis. AMA1-bearing plasmids for CRISPR/Cas9 were also used in species belonging to the closely related genus, *Penicillium*, including *P. subrubescens*[6], *P. digitatum* and *P. expansum*[7]. However, transformation efficiency was in general low (8.70-14.90%)[7], showing that further optimizations are required in these species. The limited applications of CRISPR/Cas9 in evolutionarily more distant species, including the industrial and fungal model organisms *T. reesei* and *Neurospora crassa*, respectively, is likely due to the lack of a reliable self-replicating plasmid system.

An additional advantage of CRIPSR/Cas9 genome editing using AMA1-plasmids compared to classical genetic engineering methods, is the possibility for precise marker-free modification of native genes, including point mutations, domain swapping or gene tagging and fusion. However, most studies reported the application of the CRISPR/Cas9 system for the generation of deletion strains in *A. niger*, while its application

for the introduction of other precise genetic modifications in the native fungal genes was reported to a lesser extent. In this thesis, we aimed to explore the possibilities of CRISPR/Cas9 genome editing in *A. niger*, not only by generating multi-deletion strains, but also by performing precise point mutations, fluorescent tagging or by fusing together two independent parts of different transcription factors by domain swapping.

In **Chapter 3**, based on an approach described by Nødvig et. al[35], the CRISPR/Cas9 system was used to generate transcription factor mutant strains harboring on-site specific point mutations introduced by using single-stranded oligonucleotides as repair templates. Moreover, we showed efficient introduction of single nucleotide mutations using only 60-mer oligonucleotides, further reducing the costs of template generation compared to the previously reported method using 90-mer oligonucleotides[35]. The results of this study showed that CRISPR/Cas9-mediated introduction of point mutations is a time- and cost-efficient method for the generation of strains showing increased enzyme production and activity. Our results showed that the proximity of the desired point mutation to the DNA cutting site is a crucial factor in designing the oligonucleotide repair templates. The outcome of the transformation could not be influenced by increasing the length of the repair template (up to 200 bp) when the mutated nucleotide was distant form the cutting site. Interestingly, our data showed that DNA repair was also possible with ≤12 nucleotides as flanking regions next to the DNA cutting site. This indicates that conventional 20-25 bp primers could also be used as repair templates to precisely introduce single nucleotide mutations by CRISPR/Cas9 genome editing, further reducing the costs of this method. Furthermore, micro-homology-based gene deletions using 50 bp flanking regions were also reported before in filamentous fungi[8], expanding the array of cost-efficient possibilities for the generation of DNA templates.

In **Chapter 4**, we showed that the CRISPR/Cas9 system can be used to efficiently generate a chimeric GaaR-XlnR TF by fusing together the N-terminal region of the pectinolytic TF, GaaR[36], with the C-terminal region of the xylanolytic TF, XlnR[37]. The chimeric TF showed induction of pectinolytic activities in the presence of D-xylose, offering new opportunities for enzyme production on alternative, cheap agro-industrial substrates. Generation of chimeric TFs involved in the regulation of plant biomass degradation has been reported before in *P. oxalicum*[38], *A. nidulans*[39] and *T. reesei*[40–45]. However, to our knowledge, our study was the first to report the generation of a chimeric TF by CRISPR/Cas9-mediated on-site modification of a native TF gene in a filamentous fungus.

Additionally, we used the CRISPR/Cas9 system to generate on-site edited fluorescent-

tagged versions of several endogenous TF genes. Precise fluorescent tagging of multiple native TF genes can provide new insights into the localization and interactions of key TFs during growth on plant biomass. The fluorescent tagging of the C-terminal region of three transcription factors, namely XlnR, AraR and GaaR, was performed using three different fluorescent proteins, mNeonGreen, tdTomato and mTurquoise, respectively[46]. The TF genes and the fluorescent proteins were linked by a sequence encoding a short flexible linker, GGGGS[47]. However, out of the three different fluorescently tagged mutant strains, only the GaaR:mTurquoise mutant showed slightly detectable fluorescent signal (Figure 2).

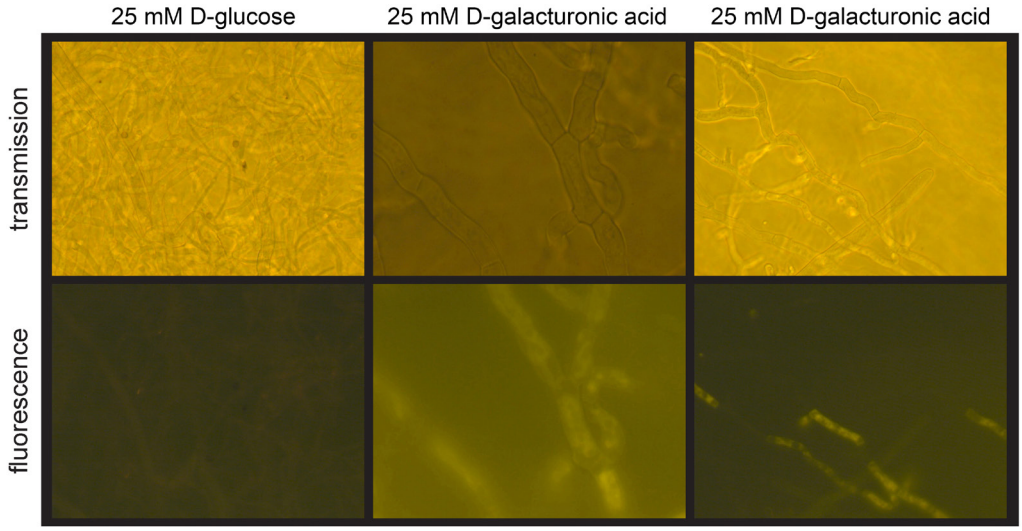


Figure 2. Screening of an *A. niger* GaaR mutant tagged with a mTurquoise fluorescent protein. Fluorescence was detected using an excitation wavelength of 450-490 nm, and emission wavelength of 525 nm. The medium containing 25 mM D-glucose represents a negative control, while the medium containing 25 mM D-galacturonic acid represents a positive control.

Moreover, the GaaR:mTurquoise and the AraR:tdTomato mutant showed impaired function, indicated by reduced growth on D-galacturonic acid (Figure 3B) and L-arabinose (Figure 3C), respectively. The colony density of the XlnR:mNeonGreen mutant was also slightly reduced compared to the control strain on beechwood xylan (Figure 3A), indicating partial loss of XlnR function as well after tagging with the fluorescent protein. Either the implementation of the fluorescent tag on the N-terminal region of the target gene, and/or the use of a different linker sequence or fluorescent protein could possibly result in the generation of TF mutants showing improved fluorescent signal and

conserved TF functionality. Although further optimization is required for this approach, tagging of native fungal genes is another possible application of the CRISPR/Cas9 system, as it was also reported in *Fusarium oxysporum*[48], *Magnaporthe oryzae*[49,50] and *A. fumigatus*[51].

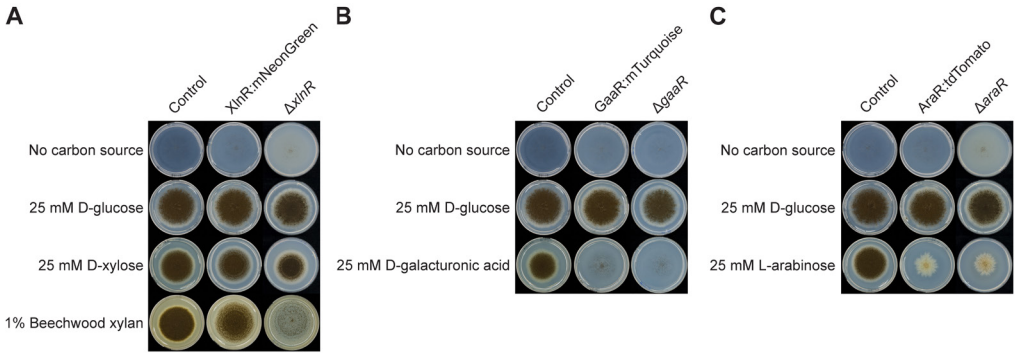


Figure 3. Growth profile of fluorescently tagged *A. niger* transcription factor mutant strains. The fluorescently tagged XlnR mutant was grown on medium containing 25 mM D-xylose or 1% beechwood xylan (**A**), the fluorescently tagged GaaR mutant was grown on medium containing 25 mM D-galacturonic acid (**B**) and the fluorescently tagged AraR mutant was grown on medium containing 25 mM L-arabinose (**C**) as substrate. Each growth test included the *A. niger* control (CBS 138852) strain as a positive control and a Δ*xlnR*, Δ*gaaR* or Δ*araR* strain as a negative control, respectively.

3. Risk assessment of the application of CRISPR/Cas9 in *Aspergillus niger*

The quick development and application of the CRISPR/Cas9 system in a wide range of species raised concerns about the possible off-target effects (e.g., random insertions or deletions) and negative consequences of its application for genome editing. Several studies have reported numerous undesired deletions, insertions or complex genomic rearrangements linked to CRISPR/Cas9 in zebrafish[52], mice[53,54] or human cell lines[54,55]. Moreover, in 2018, the Court of Justice of the European Union decided that organisms which are subject to CRISPR/Cas9 genome editing fall under the genetically modified organism (GMO) directive, thus banning the use of CRISPR/Cas9-edited crops in the agricultural sector. With respect to filamentous fungi, a thorough safety assessment regarding CRISPR/Cas9 genome editing has not been performed yet to evaluate the safety of its application in these species.

In this thesis, a large number of strains have been generated through the introduction of various mutations by CRISPR/Cas9 in *A. niger*, including multi-deletions, point mutations or domain swapping. In order to evaluate possible side-effects of CRISPR/Cas9 genome editing, a selected set of strains were subjected to whole genome sequencing (WGS). For this, we designed a control experiment, in which multiple parallel transformations have been performed in the parental *A. niger* Δ*ku70* (CBS 138852) strain using three different AMA1-plasmids: (i) a plasmid which did not carry the *cas9* gene, (ii) a plasmid carrying the *cas9* gene, but without a sgRNA expression construct, and (iii) a plasmid carrying both the *cas9* gene and a sgRNA expression construct. Based on WGS data, we assessed if the abundance of single nucleotide polymorphisms (SNPs) or random insertions and deletions (indels) could be linked to the presence and activity of the Cas9 nuclease. Results showed that the presence of Cas9 or Cas9 and sgRNA did not result in significantly increased number of undesired mutations ($p < 0.05$) (Figure 4A). Therefore, the occurred undesired mutations could rather be linked to the transformation event instead of the Cas9 activity (Figure 4A). It is worth mentioning that the transformation of an *A. niger* strain retaining an active Ku70-mediated non-homologous end joining (NHEJ) repair system with a plasmid that did not carry the *cas9* gene resulted in the accumulation of 87.75 SNPs on average. The number of SNPs further increased by up to 2.69-fold when an active CRISPR/Cas9 system was used for genome editing in this strain (data not shown). These results showed that *ku70*-defficiency results in a significantly more stable genetic background compared to its wild type in *A. niger*, highlighting the importance of background strain selection for CRISPR/Cas9-mediated transformations. It is important to note that all strains described in this thesis were derived from a *ku70*-defficient *A. niger* parental strain.

Several strains presented in **Chapter 7** and **Chapter 8** were subject to multiple rounds of transformation. Therefore, we aimed to assess the likelihood of undesired mutation accumulation in these strains. We analyzed WGS data of the sextuple deletion mutant (Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB*), and compared it to its parental strains, the quadruple Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* and double Δ*gaaR*Δ*araR* deletion mutants (all described in **Chapter 8**). Results showed that subsequent transformation events resulted in the accumulation of genomewide mutations, but to a very low extent (Figure 4B). Moreover, these mutations are more likely occurring due to the transformation event, and not due to Cas9 activity, as presented in the control experiment (Figure 4A).

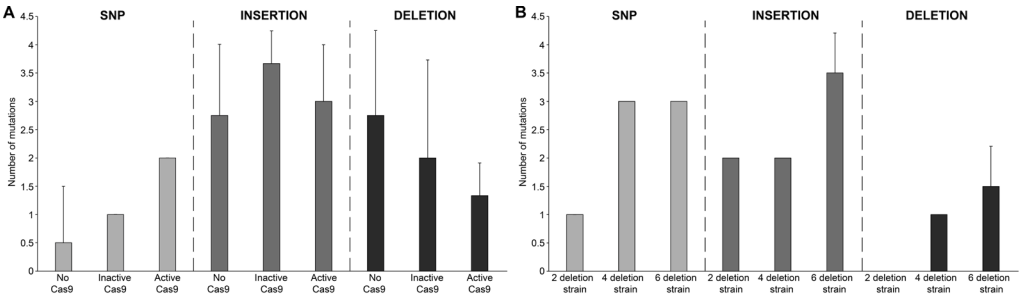


Figure 4. Risk assessment of CRISPR/Cas9 genome editing in *A. niger* Δ*ku70* strain. Graphs represent the total number of unexpected single nucleotide polymorphisms (SNP), insertions and deletions occurring in the genome of strains that were subjected to transformation events. Data was compared to the *A. niger* Δ*ku70* parental (CBS 138852) genome. **A**) Number of mutations occurring in strains transformed with a plasmid lacking the *cas9* gene (No Cas9), lacking the sgRNA expression construct (Inactive Cas9), or with a plasmid carrying both the *cas9* gene and the sgRNA expression construct (Active Cas9). **B**) Assessment of mutation accumulation after consecutive CRISPR/Cas9 transformation events. Data represents the accumulated undesired mutations after one transformation event (2 deletion strain), two transformation events (4 deletion strain) or three transformation events (6 deletion strain).

Overall, we showed that CRISPR/Cas9 is a reliable and safe technology when used in an *A. niger* Δ*ku70* strain, since unexpected mutations are most likely related to the transformation event and not to Cas9 activity. Moreover, based on the low number of accumulated mutations in the multi-deletion strains, we do not expect that undesired random mutations would affect the fitness of the strains studied in this thesis in an unexpected manner.

However, more extensive research is required to assess the safe application of CRISPR/Cas9 system across filamentous fungi. Additionally, with the improvement of sgRNA off-target assessment tools[30] and results showing safe application of CRISPR/Cas9 in cotton plants[56] or mice[57] through rational selection of optimal target sites can facilitate the public acceptance of CRISPR/Cas9 genome editing.

4. New insights into the regulation of plant biomass degradation

Filamentous fungi are primary degraders of plant biomass, and therefore, they play a pivotal role in a bio-based economy. For this, the understanding of their abilities and limits regarding plant biomass degradation is essential.

So far, a large number of *A. niger* carbohydrate active enzymes (CAZymes) involved in plant polysaccharide degradation have been identified (e.g., cellulases[58], xylanases[59] or pectinases[60]), and several key transcription factors regulating their expression (e.g., XlnR and GaaR) have been studied in detail as well. Moreover, co-regulation of genes involved in substrate degradation[61], as well as in the metabolism of sugar components[62] was previously reported. However, the regulation of the degradation of complex crude substrates and the relative contribution of TFs to this process remains poorly understood. The use of CRISPR/Ca9-mediated marker-free transformation enables the generation of multi-deletion strains suitable for the analysis of TF interactions on a network level in the process of plant biomass degradation.

In this thesis, we evaluated the contribution of major TFs to the utilization of the agro-industrial waste materials, soybean hulls, wheat bran and sugar beet pulp. Proteomic and transcriptomic data of the parental and multi-deletion strains provided new insights into the combinatorial control of gene expression and the time-dependent utilization of polysaccharide components found in crude substrates.

4.1 Preferential use of crude substrate components by *Aspergillus niger*

In **Chapter 5**, **Chapter 7** and **Chapter 8**, we assessed the ability of *A. niger* to utilize three agricultural waste materials, soybean hulls, wheat bran and sugar beet pulp, respectively. All of these substrates contain large amounts of cellulose in their compositions. Additionally, wheat bran is rich in (arabino)xylan; sugar beet pulp is rich in pectin and xyloglucan; while soybean hulls contain substantial amounts of (galacto) mannan next to xyloglucan and pectin. The analysis of omics data from *A. niger* Δ*ku70* strain (CBS 138852) showed the adaptation of *A. niger* to the utilization of the major components in each substrate in a timely manner.

Transcriptomic data originated from cultures grown for 2 h on soybean hulls (**Chapter 5**) and sugar beet pulp (**Chapter 8**) showed the highest expression of the α-glucosidase-encoding gene *agdB*, and the exo-inulinase-encoding gene *inuE*, respectively. Although, sugar beet pulp might contain low levels of residual sucrose in its composition, the high expression of *agdB* on soybean hulls was unexpected. This can possibly be explained by the induction of AmyR in the presence of trace amounts of inducing components[63], such as D-glucose[64]. Similarly, in **Chapter 5**, transcriptomic data originated from 2 h cultivation of *A. niger* on guar gum also showed the highest expression of four

amylolytic (*glaA*, *agdA*, *aamA* and *agdB*) and one inulinolytic (*inuE*) gene, as well as 4-4.5-fold higher expression of the major transcriptional repressor gene *creA*, compared to later timepoints. CreA activity in the early stages of growth has been reported before in this fungus when grown on crude substrates[65]. Generally, these data showed that the induction of the amylolytic and inulinolytic system is predominant in the early stages of growth in *A. niger*, even if the inducers of these systems are present only in trace amounts. Moreover, early CreA activity can be linked to the presence of D-glucose and D-fructose released by the amylolytic and inulinolytic enzymes.

After 8 h of growth on soybean hulls (**Chapter 5**) and sugar beet pulp (**Chapter 8**), the expression of pectinolytic genes was predominant (28 and 34 pectinolytic genes, respectively). Although, both substrates are rich in pectin and xyloglucan as well, *A. niger* appears to respond to the presence of pectin at an earlier stage of growth. This preference is also indicated by the large number of pectinolytic genes found in the *A. niger* genome[66]. Most hemicellulolytic genes (coding for xyloglucanases, (arabino) xylanases or (galacto)mannanases) were mainly expressed in the later stages of growth, after 8 or 24 h of cultivation in these studies. Finally, the expression of cellulolytic genes showed high upregulation after 24 h of cultivation compared to earlier timepoints.

In **Chapter 7**, proteomic data originated from 24 h wheat bran cultures showed the most abundant presence of the β-1,4-endoxylanase XlnC/XynA and the arabinoxylan arabinofuranohydrolase AxhA, followed by several cellulolytic proteins. This indicates the preference for the degradation of (arabino)xylan over cellulose in wheat bran by *A. niger*.

Overall, omics data from all three studies indicate slow utilization of cellulose by *A. niger*, despite its abundant presence in each tested crude substrate. Moreover, slow utilization of cellulose is also supported by the growth profile results of these studies.

In conclusion, data described in this thesis provide more understanding about the ability of *A. niger* to adapt to the degradation polysaccharides found in agro-industrial materials, and can facilitate the selection of suitable substrates for industrial applications.

4.2 Contribution of transcription factors to the regulation of plant biomass utilization

The roles of several TFs involved in plant biomass utilization, including XlnR[37], AraR[67], GaaR[36], RhaR[68] or AmyR[64] have been studied to a large extent in *A. niger*. However,

only a few reports have described the roles of ClrA[69], ClrB[69], InuR[70] and GalX[71] in this process. In this thesis, we expanded the knowledge about several major regulators of plant biomass utilization by analyzing both their individual impact on substrate utilization, as well as their roles within the regulatory network.

In **Chapter 5**, using transcriptomic data, we showed that mannobiose is most likely the inducer of ClrB in *A. niger*. Moreover, gene expression data from guar gum and soybean hulls cultures showed the control of ClrB over the expression of several (galacto)mannanase-, cellulase-, xyloglucanase- and transporter-encoding genes. Overall, data showed that the role of *A. niger* ClrB is comparable to the role of ClrB in *A. oryzae*[72], while its regulon is different from that of the ClrB ortholog described in *A. nidulans*[73]. Interestingly, the growth impairment of the Δ*clrB* mutant on soybean hulls was comparable to the growth defect of a Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB*Δ*amyR* quintuple deletion mutant on wheat bran or a Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*Δ*xlnR*Δ*clrB* sextuple deletion mutant on sugar beet pulp. The fact that ClrB does not control the expression of major pectinolytic or xylanolytic genes indicates that most likely the degradation of (galacto)mannan is crucial during soybean hulls utilization.

Additionally, in **Chapter 7**, proteomic data demonstrated a major contribution of ClrB to the utilization of wheat bran, while in **Chapter 8**, the single deletion of *clrB* resulted in the highest growth defect when grown on solid medium containing sugar beet pulp as substrate. However, the dominant role of ClrB in sugar beet pulp utilization could not be supported by exoproteome or gene expression analysis of liquid culture samples. Similar results were observed in **Chapter 6**, where data indicated the involvement of AmyR in inulin utilization, only demonstrated by data originated from cultures grown on solid media. These results support the observation that phenotypes of solid and liquid cultures cannot be directly compared[74]. Although studying the characteristics of submerged fungal cultures is valuable for industrial applications, these do not fully represent the natural behavior of the fungus.

The involvement of ClrA in the regulation of (hemi-)cellulose utilization was assessed in **Chapter 7**. The analysis of enzyme activity assay and proteomic results indicated only partial involvement of ClrA in cellulose degradation. Although, the deletion of *clrA* resulted in decreased β-glucosidase (BGL) activity, and it reduced the production of three cellobiohydrolases (CbhA, CbhB and CbhC), it did not fully abolish growth on cellulose. Interestingly, ClrA showed partial control over β-xylosidase (BXL) activity, however, this was overruled by XlnR. In **Chapter 5**, we showed that the expression of *clrA* is (partially) dependent on ClrB. Therefore, it cannot be excluded that a part of the

genes associated with the ClrB regulon are in fact controlled by ClrA.

In **Chapter 6**, compared to previous literature[70], we identified additional genes under the control of InuR, while in **Chapter 8** we concluded that GalX does not control the expression of CAZy-encoding genes, and its role is restricted only to the regulation of sugar metabolism[75].

4.3 Insights into gene co-regulation and transcription factor interactions

The generation and analysis of combinatorial deletion mutants allow for the identification of co-regulated genes, and can reveal TF interactions during the process of plant biomass utilization. The co-regulation of CAZy-encoding genes in *A. niger* was previously studied only to a limited extent[61,76].

In **Chapter 7**, the combined analysis of (hemi-)cellulolytic regulators revealed several cases of gene co-regulation. For instance, the β-galactosidase (LAC) activity was not abolished in any of the single deletion mutants (Δ*xlnR*, Δ*araR*, Δ*clrA* or Δ*clrB*). In contrast, each double mutant with a deleted *xlnR* showed abolished LAC activity. Similarly, proteomic results showed the abolished production of the β-galactosidase LacA, which was only observed in the exoproteome of the quadruple Δ*xlnR*Δ*araR*Δ*clrA*Δ*clrB* mutant. Additionally, the single deletion of *clrA* indicated partial control of BGL and BXL activity by ClrA. However, data showed that XlnR could fully compensate for the loss of ClrA function in several double and triple deletion strains. In contrast, the single deletion of *clrB* showed an unexpected growth improvement on wheat bran, which indicates no role for this TF in wheat bran utilization. However, the analysis of combinatorial deletion strains showed that ClrB plays a major role within the regulatory network governing wheat bran degradation. These results showed the importance of the analysis of combinatorial TF deletion strains in order to assess the relative role of each TF within the regulatory network governing plant biomass degradation.

Regarding TF interactions, our data provided more information about the interaction of the (hemi-)cellulolytic regulators XlnR, ClrA and ClrB. Previously, different activation patterns have been reported for ClrA/CLR-1 and ClrB/CLR-2 in *N. crassa* and *Aspergillus* species[77]. Moreover, it has also been shown that XlnR controls the expression of *clrA* and *clrB* in *A. niger*[69], but no interaction between ClrA and ClrB was suggested in this species. In contrast with the CLR-1 dependent activation of CLR-2

observed in *N. crassa*[78], in **Chapter 5**, we showed that the expression of the *A. niger clrA* depends on ClrB activity. Based on proteomic data analyzed in **Chapter 7**, we showed that XlnR contributed the most to wheat bran degradation, which was followed by ClrB and ClrA, in the corresponding hierarchical order. The relative contribution of these three TFs to the regulation of (hemi-)cellulose utilization could partly be explained by sequential activation of ClrA by ClrB, which is in turn regulated by XlnR. Therefore, our data provides a better understanding of TF interactions within the (hemi-)cellulolytic regulatory network of *A. niger*.

Previous studies suggested antagonistic interactions between the (hemi-)cellulolytic regulator XlnR and the pectinolytic regulators AraR[79] and GaaR[61]. In **Chapter 8**, we showed evidence of the upregulation of (hemi-)cellulolytic genes when the utilization of pectin was impaired in the Δ*araR*, Δ*gaaR* and Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* mutants. Moreover, the increased expression of (hemi-)cellulolytic genes was also supported by the highly increased expression of *clrB*. This shows that the antagonistic effect is not only restricted to the interactions of XlnR and AraR/GaaR, but involves multiple TFs involved in the complex regulatory network of (hemi-)cellulose and pectin utilization.

The ability of *A. niger* to adapt its regulatory network to the utilization of alternative components when the utilization of major polysaccharides is impaired suggests that this fungus possesses a flexible regulatory network controlling plant biomass utilization. This is likely a major reason for the high suitability of *A. niger* for various industrial applications.

5. Identification of novel transcription factors involved in plant biomass utilization

The genome of *A. niger* contains over 500 putative transcription factor-encoding genes, of which only nine major transcriptional activators (XlnR, ClrA, ClrB, GaaR, AraR, RhaR, GalX, AmyR and InuR) and two repressors (CreA and GaaX) have been identified to be involved in the regulation of plant polysaccharide utilization. These TFs show diverse rate of conservation across species. For instance, XlnR is conserved in nearly all filamentous ascomycetes studied so far, while AraR appears to be conserved only in the order of Eurotiales[80]. Moreover, the function of conserved TFs can also differ to a varying extent across species. For instance, CLR-2/ClrB is a conserved TF which was reported in *N. crassa*[78] and several *Aspergillus*[69,72,78] species. However, it shows

distinct induction pattern between *N. crassa* and *Aspergillus* species[77], and its regulon differs even between the closely related *A. niger* and *A. nidulans*[73]. Nevertheless, the availability of genome sequences has promoted the identification of novel TFs in different species based on phylogenetic analysis.

A previous study reported the identification of a transcriptional activator of cellulose utilization in *N. crassa*, identified as CLR-4[81]. Based on sequence homology, we predicted the NRRL3_04942 (ClrD) as a putative ortholog of *N. crassa* CLR-4, with possible involvement in cellulose utilization. Similarly, we investigated the role of a putative endoxylanase repressor, NRRL3_00417, predicted based on sequence similarity to the *T. reesei* SxlR[82]. Besides the phylogenetic approach, a machine learning-based approach[83] that evaluated gene expression profiles originated from large gene expression datasets generated in this thesis also suggested putative roles in plant biomass utilization for the ClrD and SxlR candidates in *A. niger* (data not shown).

The CRISPR/Cas9 system was used to delete both candidate TFs in the *A. niger* Δ*ku70* (CBS 138852) strain, and the phenotype of the deletion mutant strains was compared to the *A. niger* parental strain on several substrates related to their expected functions. However, based on initial characterization, no function related to (hemi-)cellulose utilization could be associated with ClrD in *A. niger* (Figure 5A). Similarly, the deletion of the *sxlR* candidate did not result in the expected increase of xylanase activity when cultivated on wheat bran or beechwood xylan (Figure 5B), and the growth of the Δ*sxlR* mutant was not affected either on these substrates (data not shown). The Δ*sxlR* mutant showed statistically significant increase of endoxylanase activity only when cultivated on beechwood xylan for 24 h (Figure 5B). However, the increase of activity was minimal, and the selection of a stricter statistical cut-off value ($p < 0.01$) would result in no significant differences between the control and mutant strain.

Interestingly, in **Chapter 6** and **Chapter 8**, transcriptomic data showed high expression of several putative pectinolytic genes (*abnC*, NRRL3_10498 and NRR3_3855), when the substrate utilization was impaired. This could be a result of culture stress, however, the possibility of the activation of a regulatory back-up system for substrate utilization mediated by unknown transcription factors cannot be excluded. Although *in silico* prediction of TF genes is necessary for the identification of novel TFs, experimental validation (e.g., generation and analysis of deletion strains) needs to be performed to identify these genes.

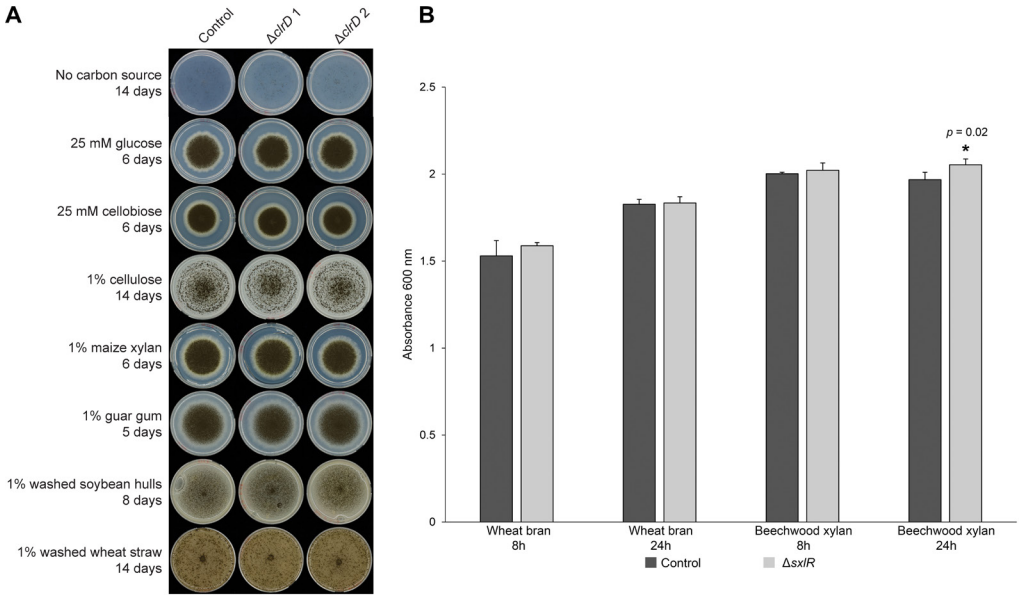


Figure 5. Phenotypic screening of two putative transcription factor deletion strains. A) Growth profile results of *A. niger* control (CBS 138852) and two independent Δ*clrD* (*N. crassa clr-4* ortholog) strains. Each plate was inoculated with 1000 freshly harvested conidia, and grown at 30°C for up to 14 days. Each plate was inoculated in technical duplicates. **B**) Endoxylanase activity assay of *A. niger* control (CBS 138852) and Δ*sxlR* (*T. reesei sxlR* ortholog) strain. Cell-free culture supernatant samples originated from 1% wheat bran or 1% beechwood xylan after 8 or 24 h incubation in a rotary shaker at 30°C and 250 rpm were used for this assay. Activity is represented as relative absorbance value measured at 600 nm. Statistically significant (p <0.05) difference between the control and mutant strain is indicated by (*).

6. Concluding remarks and future work

The regulation of plant biomass degradation in *A. niger* is controlled by a complex and intricate system involving the interaction of several TFs in this process. The individual roles of several major TFs have previously been described, however, their roles within the regulatory network controlling the degradation of complex agricultural waste materials remain poorly understood. The analysis of deletion mutants generated in **Chapter 7** and **Chapter 8** contributes to the understanding of the combinatorial gene expression for the utilization of two agro-industrial substrates, wheat bran and sugar beet pulp, respectively. Additionally, the analysis of omics data showed the adaptation of *A. niger* to the utilization of substrate components, even when the degradation of major polysaccharides was blocked. Moreover, the deletion of major TFs involved in polysaccharide utilization can reveal putative back-up regulatory mechanisms, which

can possibly indicate the action of novel TFs involved in this process. The generation and analysis of a strain carrying the deletion of all nine major transcriptional activators analyzed in this thesis could provide more information about such back-up regulatory functions in *A. niger*. Moreover, the combined deletion of TFs regulating the expression of CAZy-encoding genes as well as the deletion of the protease regulator gene *prtT* can result in strains suitable for cell factories by showing minimal background protein production.

The application of the CRISPR/Cas9 system for the introduction of various precise mutations in native *A. niger* genes showed the possibility to time- and cost-efficiently engineer strains showing improved (**Chapter 3**) or altered CAZyme production (**Chapter 4**). The utilization of CRISPR/Cas9 system enables new possibilities for further improvement of CAZyme producer strains. For instance, production could be improved by placing a constitutively active chimeric TF under the control of a strong promoter, such as *gpdA*[84]. Moreover, on-site fluorescent-tagging of chimeric TFs could provide more understanding about their localization and functions, however, more improvements need to be performed in this regard.

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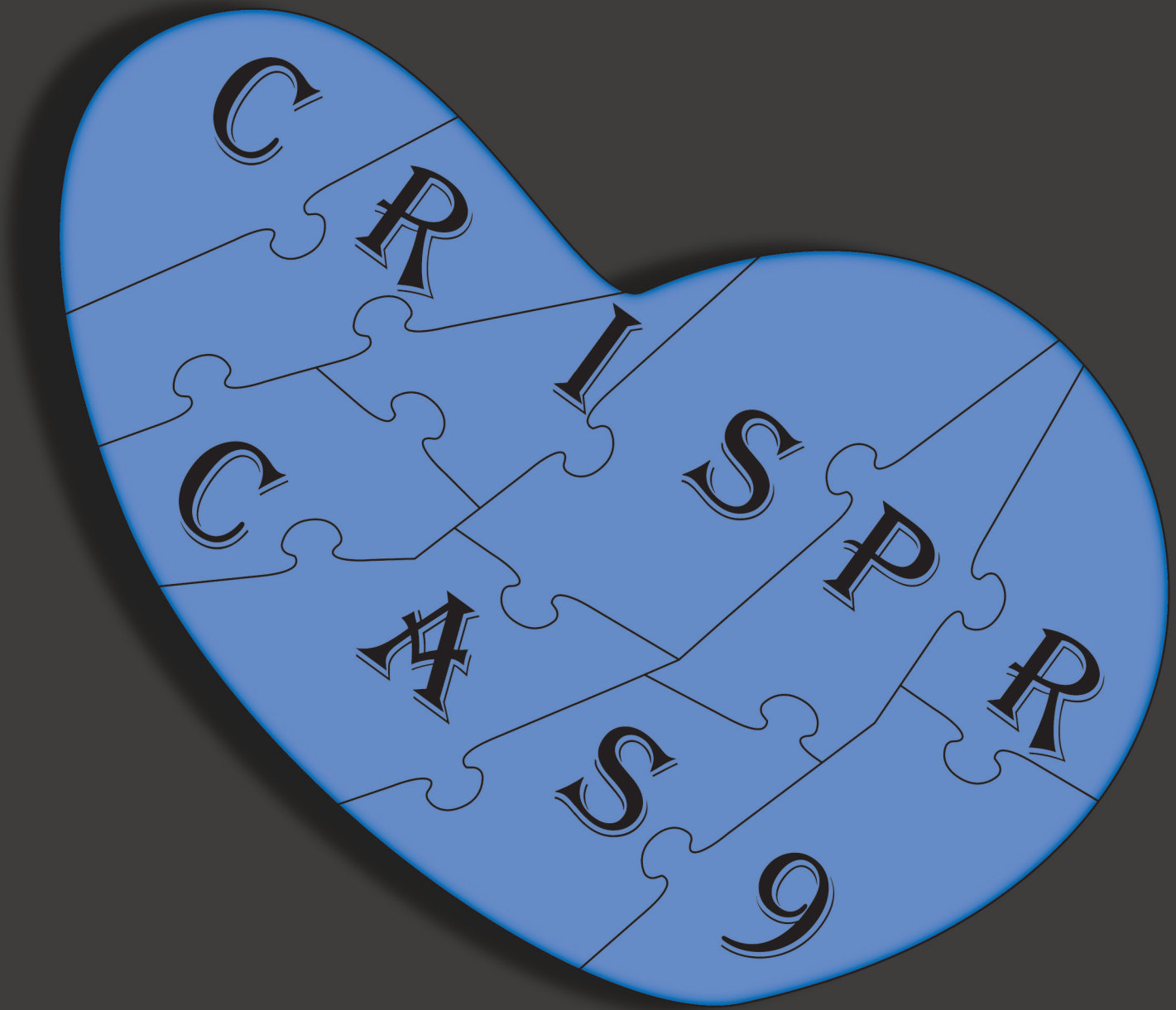
CRISPR
CAS9

Appendix

Het toepassen van het CRISPR/Cas9-systeem om het regulatie netwerk van plantenbiomassa afbraak in *Aspergillus niger* te bestuderen

Het CRISPR/Cas9-systeem is in 2012 ontwikkeld als hulpmiddel voor het bewerken van genen en wordt sindsdien voor verschillende doeleinden toegepast in een breed scala van organismen, waaronder filamenteuze schimmels (**hoofdstuk 2**).

Filamenteuze schimmels zijn primaire afbrekers van plantaardige biomassa en spelen daarom een cruciale rol in de biobased economy. Hiervoor is het begrijpen van hun mogelijkheden en limities met betrekking tot de afbraak van plantenbiomassa essentieel. De regulatie van de afbraak van plantenbiomassa in *Aspergillus niger* wordt gecontroleerd door een complex en ingewikkeld systeem waarbij de interactie van meerdere transcriptiefactoren (TF's) betrokken is die elk de expressie reguleren van genen die coderen voor koolhydraat afbrekende enzymen (CAZyme). De individuele rol van verschillende belangrijke TF's (bijv. XlnR en GaaR) zijn eerder beschreven, maar hun rol binnen het regulerende netwerk dat de afbraak van complexe agro-industriële afvalstoffen controleert, blijven slecht begrepen. Hoewel klassieke gendeleties met homologe recombinatie op grote schaal werden uitgevoerd in *A. niger* om TF's te bestuderen, waren deze methoden beperkt met betrekking tot precieze modificaties van de endogene TF-coderende genen.

In dit proefschrift werd CRISPR/Cas9-genbewerking zonder markers uitgevoerd om precieze puntmutaties te genereren (**hoofdstuk 3**), domeinuitwisseling (**hoofdstuk 4**) en gentagging door fluorescerende markers die zich richten op verschillende belangrijke TF's die de afbraak van plantenbiomassa coördineren. In **hoofdstuk 3** hebben we de efficiënte introductie van enkelvoudige nucleotide mutaties in de *xlnR*- en *gaaR*-genen laten zien met zeer korte als reparatietemplates, tot 60-meer-oligonucleotiden. De resultaten van deze studie toonden aan dat CRISPR/Cas9-gemedieerde introductie van puntmutaties een tijd- en kostenefficiënte methode is voor het genereren van stammen die verhoogde enzymproductie en activiteit vertonen. In **hoofdstuk 4** hebben we laten zien dat het CRISPR/Cas9-systeem kan worden gebruikt om op efficiënte wijze een chimere GaaR-XlnR TF te genereren, door het N-terminale gebied van de pectinolytische TF, GaaR, te fuseren met het C-terminale gebied van de xylanolytische TF, XlnR. De chimere TF toonde inductie van pectinolytische activiteiten in de aanwezigheid van D-xylose, wat nieuwe mogelijkheden biedt voor enzymproductie op alternatieve, goedkope agro-industriële substraten.

Bovendien werden markervrije CRISPR/Cas9-transformaties uitgevoerd om de beperkingen van klassieke transformatiemethoden met betrekking tot het aantal gendeleties te overwinnen. We toonden het genereren van enkele (**hoofdstuk 5**) of dubbele (**hoofdstuk 6**) deletiestammen in een enkele transformatiegebeurtenis, of multi-deletiestammen (**hoofdstuk 7 en 8**) door opeenvolgende transformatiegebeurtenissen. Door CRISPR/Cas9 gemedieerde transformatie met behulp van het AMA1-dragende plasmide en de daaropvolgende eliminatie door tegenselectie liet geen beperkingen zien met betrekking tot het aantal genen dat kan worden verwijderd in *A. niger*.

Bovendien leverde de analyse van transcriptomische of proteomische gegevens afkomstig van de ouder- en (multi-)deletiestammen van *A. niger* op agro-industriële afvalstoffen meer inzicht in het preferentiële gebruik van substraatcomponenten. In **hoofdstuk 5**, **hoofdstuk 7** en **hoofdstuk 8** hebben we het vermogen van *A. niger* onderzocht om drie agro-industriële afvalmaterialen, respectievelijk sojabonenschillen, tarwezemelen en suikerbietenpulp, te gebruiken. Al deze substraten bevatten grote hoeveelheden cellulose. Daarnaast zijn tarwezemelen rijk aan (arabino)xylaan, suikerbietenpulp is rijk aan pectine en xyloglucaan, terwijl sojabonen naast xyloglucaan en pectine aanzienlijke hoeveelheden (galacto)mannaan bevatten. De analyse van omics-gegevens onthulde de efficiënte aanpassing van *A. niger* aan het gebruik van de belangrijkste componenten in elk substraat, zelfs wanneer de afbraak van de belangrijkste polysachariden werd geblokkeerd.

De gegevens die in dit proefschrift zijn gegenereerd, de kennis uitgebreid over de individuele rol van en interacties tussen verschillende belangrijke TF's die betrokken zijn bij de regulatie van het gebruik van plantenbiomassa. In **hoofdstuk 5** hebben we met behulp van transcriptomische data laten zien dat mannobiose de inductor is van ClrB in *A. niger*. Bovendien toonden genexpressiegegevens van cultures op guar gum en sojabonen de controle van ClrB aan over de expressie van verschillende (galacto) mannanase-, cellulase-, xyloglucanase- en transporter-coderende genen. Daarnaast toonden in **hoofdstuk 7** proteomische gegevens een belangrijke bijdrage van ClrB aan het gebruik van tarwezemelen, terwijl in **hoofdstuk 8** een vergelijkende groeianalyse van meerdere enkelvoudige TF-deletiestammen aantoonde dat de deletie van clrB resulteerde in de grootste groeivermindering wanneer gegroeid werd op vast medium met suikerbietenpulp als substraat. Deze gegevens tonen de belangrijke rol van ClrB bij de afbraak van verschillende ruwe substraten met diverse samenstellingen.

De generatie en analyse van combinatorische deletiemutanten maakte de identificatie van co-gereguleerde genen mogelijk en onthulde TF-interacties tijdens de omzetting

van plantenbiomassa. In **hoofdstuk 7** onthulde de gecombineerde analyse van (hemi-)cellulolytische regulatoren verschillende gevallen van co-regulatie van genen en de relatieve rol van elke TF binnen het regulerende netwerk dat de afbraak van tarwezemelen regelt. Met betrekking tot TF-interacties hebben we in **hoofdstuk 5** laten zien dat de expressie van *A. niger clrA* afhankelijk is van ClrB-activiteit. Bovendien toonden de proteomische data geanalyseerd in **hoofdstuk 7** aan dat XlnR het sterkst bijdroeg aan de afbraak van tarwezemelen, gevolgd door ClrB en ClrA, in de overeenkomstige hiërarchische volgorde. De relatieve bijdrage van deze drie TF's aan de regulering van het gebruik van (hemi-)cellulose kan gedeeltelijk worden verklaard door de opeenvolgende activering van ClrA door ClrB, dat op zijn beurt wordt gereguleerd door XlnR. Daarom bieden onze gegevens een beter begrip van TF-interacties binnen het (hemi-)cellulolytische regulerende netwerk van *A. niger*.

Eerdere studies suggereerden antagonistische interacties tussen de (hemi-) cellulolytische regulator XlnR en de pectinolytische regulatoren AraR en GaaR. In **hoofdstuk 8** hebben we bewijs geleverd van de opregulatie van (hemi-)cellulolytische genen wanneer het gebruik van pectine verminderd was in de Δ*araR*-, Δ*gaaR*- en Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX*-mutanten. Bovendien werd de verhoogde expressie van (hemi-)cellulolytische genen ook ondersteund door de sterk verhoogde expressie van *clrB*. Dit toont aan dat het antagonistische effect niet alleen beperkt is tot de interacties van XlnR en AraR/GaaR, maar ook betrekking heeft op meerdere TF's die betrokken zijn bij het complexe regulerende netwerk van (hemi-)cellulose en pectinegebruik. Het vermogen van *A. niger* om zijn regulerend netwerk aan te passen aan het gebruik van alternatieve componenten wanneer het gebruik van belangrijke polysachariden wordt geblokkeerd, suggereert dat deze schimmel een flexibel regulerend netwerk bezit dat het gebruik van plantenbiomassa regelt. Dit is waarschijnlijk een belangrijke reden voor de hoge geschiktheid van *A. niger* voor verschillende industriële toepassingen.

Ten slotte ondersteunen de gegevens beschreven in dit proefschrift ook de observatie dat fenotypes van vaste en vloeibare culturen niet direct kunnen worden vergeleken. In **hoofdstuk 8**, ondanks de sterke groeivermindering veroorzaakt door de deletie van *clrB* wanneer gekweekt op vast medium dat suikerbietenpulp bevatten, kon de dominante rol van ClrB in het gebruik van dit substraat niet worden ondersteund door exoproteoom- of genexpressie-analyse van vloeibare culturen. Vergelijkbare resultaten werden waargenomen in **hoofdstuk 6**, waar gegevens de betrokkenheid van AmyR bij het gebruik van inuline aantoonden, alleen kwamen van kweken op vaste media. Hoewel het bestuderen van de kenmerken van vloeibare schimmelculturen waardevol is voor industriële toepassingen, vertegenwoordigen deze niet het natuurlijke gedrag

van de schimmel.

Samengevat laten de resultaten die in dit proefschrift worden beschreven zien dat verschillende toepassingen van het CRISPR/Cas9-systeem voor de ontwikkeling van TF's diede regulatie van het gebruik van plantenbiomassa controleren, en de analyse van verschillende TF's op netwerkniveau in *A. niger* mogelijk maakten. Bovendien kan de deletie van belangrijke TF's die betrokken zijn bij het gebruik van polysachariden vermeende back-upregulerende mechanismen aan het licht brengen, die mogelijk kunnen wijzen op de werking van nieuwe TF's die bij dit proces zijn betrokken. Bovendien kan de gecombineerde deletie van TF's die de expressie van CAZyme-coderende genen reguleren, evenals de deletie van het protease-regulatorgen *prtT* resulteren in stammen die geschikt zijn voor celfabrieken met een verminderde productie van achtergrondeiwit.

A CRISPR/Cas9 rendszer alkalmazása az *Aspergillus niger* növényi biomassza lebontását irányító regulációs hálózatának tanulmányozására

A CRISPR/Cas9 rendszert először 2012-ben alkalmazták génszerkesztő eszközként, és azóta is különféle célokra használták élőlények széles körében, beleértve a fonalas gombákat is (**2. fejezet**).

A fonalas gombák a növényi biomassza elsődleges lebontói, ezért kulcsszerepet játszanak a bioalapú gazdaságban. Ehhez elengedhetetlen a növényi biomassza lebontásával kapcsolatos képességeik és határaik megértése. A növényi biomassza lebontását *Aspergillus niger*-ben egy komplex rendszer szabályozza, amely magában foglalja a szénhidrát aktív enzimeket (CAZyme) kódoló gének expresszióját szabályozó transzkripciós faktorokat (TF). Számos kulcsfontosságú TF (például XlnR és GaaR) egyéni szerepét korábban leírták, azonban funkciójuk a komplex összetételű agráripari hulladékanyagok lebontását irányító regulációs hálózaton belül továbbra sem teljesen ismert. Az *A. niger*-ben széles körben végeztek szelekciós marker alapú homológ-rekombinációval járó klasszikus géndeléciókat a TF-ek tanulmányozására, azonban hasonló transzformációs metódusok korlátozottak voltak az endogén TF-eket kódoló gének precíz módosításainak céljára.

Ebben a kutatásban számos markermentes CRISPR/Cas9 génszerkesztést végeztünk, beleértve precíz pontmutációkat (**3. fejezet**), doméncserét (**4. fejezet**) és gének fluoreszcens markerekkel való jelölését, amelyek számos növényi biomassza lebomlását irányító TF-eket érintettek. A **3. fejezetben** egyetlen nukleotid mutációk hatékony bevezetését mutattuk be az *xlnR* és *gaaR* génekben, akár 60 bázisból álló oligonukleotidok használatával. A tanulmány eredményei azt mutatták, hogy a markermentes CRISPR/Cas9 transzformálás egy idő- és költséghatékony módszer fokozott enzimtermelést és aktivitást mutató törzsek létrehozására, egyszerű pontmutációk bevezetése által. A **4. fejezetben** megmutattuk, hogy a CRISPR/Cas9 rendszer hatékonyan használható egy GaaR-XlnR kiméra TF létrehozására, a pektinolitikus TF, GaaR N-terminális régiójának és a xilanolitikus TF, XlnR C-terminális régiójának kombinálásával. A kiméra TF pektinolitikus aktivitást mutatott D-xilóz jelenlétében, új lehetőségeket kínálva az enzimtermeléshez, olcsó alternatív agráripari szubsztrátumokon.

Ezeken kívül, további markermentes CRISPR/Cas9 transzformációkat végeztünk deléciós mutánsok generálására is, leküzdve a klasszikus transzformációs módszerek korlátait a géndeléciók számát illetően. Megmutattuk az egyszeres (**5. fejezet**) vagy a dupla (**6. fejezet**) deléciós törzsek létrehozását egyetlen transzformálás által, vagy a többszörös deléciós törzsek (**7. és 8. fejezet**) létrehozását több transzformációs lépés által. Az AMA1-et hordozó plazmid alapú CRISPR/Cas9 transzformálás által, majd ennek a plazmidnak a kontraszelekcióval történő eliminálásával nem tapasztaltunk korlátokat az *A. niger*-ben deletálható gének számát illetően.

Ezen eredményeken kívül, az agráripari hulladékanyagokon növesztett *A. niger* szülői és (multi-)deléciós törzsekből származó transzkriptomikai vagy proteomikai adatok elemzése betekintést nyújtott a szubsztrátum komponensek preferenciális használatába. Az **5.**, **7.** és **8. fejezetben** felmértük az *A. niger* képességét három mezőgazdasági hulladékanyag, a szójabab héj, a búzakorpa és a cukorrépapép hasznosítására. Ezek a szubsztrátumok nagy mennyiségű cellulózt tartalmaznak összetételükben. Ráadásul, a búzakorpa (arabino)xilánban, a cukorrépapép pektinben és xiloglukánban gazdag, míg a szójabab héj a xiloglukán és a pektin mellett jelentős mennyiségű (galakto)mannánt is tartalmaz. Az omikai adatok elemzése bemutatta, hogy az *A. niger* az idő folyamán alkalmazkodott az egyes szubsztrátumok komponenseinek hasznosításához, akkor is, ha a főbb poliszacharidok lebomlását irányító regulációs hálózat blokkolva volt.

Ebben a tézisben leírt adatok bővítették a növényi biomassza lebontás szabályozásában részt vevő jelentősebb TF-ek egyéni szerepeiről és kölcsönhatásairól szóló ismereteket. Az **5. fejezetben** transzkriptomikai adatok felhasználásával bemutattuk, hogy *A. niger*-ben a mannobióz indukálja a ClrB TF-t. A guargumin és szójabab héjon növesztett tenyészetekből származó transzkriptomikai adatok azt mutatták, hogy a ClrB szabályozza számos (galakto)mannanáz-, celluláz-, xiloglukanáz- és transzporter-kódoló gének expresszióját. Ezenkívül, a **7. fejezetben**, a proteomikai adatok azt mutatták, hogy a ClrB jelentős mértékben hozzájárul a búzakorpa hasznosításához. Ráadásul, a **8. fejezetben** több TF deléciós törzseknek a komparatív növekedési kísérlete azt mutatta, hogy a *clrB* deléciója eredményezte a legszignifikánsabb növekedés csökkenést cukorrépapépet tartalmazó szilárd táptalajon. Ezek az adatok igazolják a ClrB fontos szerepét számos különböző összetételű nyers agráripari szubsztrátum lebontásában.

A kombinatorikus deléciós mutánsok generálása és elemzése lehetővé tette a ko-regulált gének azonosítását, és feltárt számos TF kölcsönhatásokat a növényi biomassza hasznosításának folyamata során. A **7. fejezetben** a (hemi)cellulolitikus regulátorok kombinált elemzése gének ko-regulációjának számos esetét és az egyes

TF-ek relatív szerepét tárta fel a búzakorpa lebomlását szabályozó hálózaton belül. A TF kölcsönhatásokat illetően az **5. fejezetben** megmutattuk, hogy az *A. niger clrA* expressziója a ClrB aktivitásától függ. Ezenkívül, a **7. fejezetben** elemzett proteomikai adatok azt mutatták, hogy leginkább az XlnR járult hozzá a búzakorpa lebontásához, amelyet a ClrB és a ClrA követett, a megfelelő hierarchikus sorrendben. Ennek a három TF-nek a relatív hozzájárulása a (hemi)cellulóz hasznosítás szabályozásához részben a ClrA-nak a ClrB általi szekvenciális aktiválásával magyarázható, amelyet viszont az XlnR szabályoz. Következésképpen, adataink jobban rávilágítanak az *A. niger* (hemi) cellulolitikus szabályozó hálózatán belüli TF kölcsönhatásokra.

Korábbi tanulmányok antagonista kölcsönhatásokat javasoltak a (hemi)cellulolitikus regulátor XlnR és a pektinolitikus regulátorok AraR és GaaR között. A **8. fejezetben** bemutattuk a (hemi)cellulolitikus gének expressziójának növekedését, amikor a pektin hasznosulása korlátozva volt a Δ*araR*, Δ*gaaR* és a Δ*gaaR*Δ*araR*Δ*rhaR*Δ*galX* mutánsokban. Ezenkívül, a (hemi)cellulolitikus gének fokozott expresszióját a *clrB* expressziójának növekedése is támogatta. Ez azt mutatja, hogy az antagonista hatás nem csak az XlnR és az AraR/GaaR kölcsönhatásaira korlátozódik, hanem több TF-t is érint, amelyek részt vesznek a (hemi)cellulóz és pektin hasznosítás komplex szabályozási hálózatában. Az *A. niger* képessége, hogy a regulációs hálózatát az alternatív komponensek hasznosításához tudja igazítani, amikor a főbb poliszacharidok lebontása korlátozott, arra utal, hogy ez a gomba egy rugalmas szabályozási hálózattal rendelkezik a növényi biomassza hasznosítására. Valószínűleg, ez az egyik fő oka annak, hogy az *A. niger* kiválóan alkalmas különféle ipari alkalmazásokra.

Végül, az ebben a tézisben ismertetett adatok alátámasztják azt a megfigyelést is, hogy a szilárd és folyékony kultúrák fenotípusai nem közvetlenül összehasonlíthatóak. A **8. fejezetben** a *clrB* deléciója által okozott, cukorrépapépet tartalmazó szilárd kultúrákon megfigyelt nagymértékű növekedés csökkenés ellenére, a ClrB domináns szerepe a cukorrépapép hasznosításában nem volt alátámasztható a folyékony kultúrákból származó adatokkal. Hasonló eredményeket figyeltünk meg a **6. fejezetben** is, ahol az adatok az AmyR szerepét jelezték az inulin hasznosításban, amit csak a szilárd táptalajon növesztett tenyészetekből származó adatok igazoltak. Habár a folyékony kultúrák jellemzőinek tanulmányozása értékes az ipari alkalmazásokhoz, ezek nem reprezentálják a gomba természetes viselkedését.

Összességében, ezen dolgozatban ismertetett eredmények bemutatták a CRISPR/Cas9 rendszer különféle alkalmazásait a növényi biomassza hasznosítását szabályozó TF-ek módosítására, és lehetővé tették több TF hálózati szintű elemzését *A. niger*-

ben. Ezen túlmenően, a poliszacharid hasznosításban részt vevő fő TF-ek deléciója további, új TF-ek szerepét tárhatja fel a szabályozási hálózatban. Végül, a CAZyme-t kódoló gének expresszióját szabályozó TF-ek kombinált deléciója, valamint a proteáz regulátort kódoló gén, *prtT* további deléciója olyan korlátozott háttérfehérje-termeléssel rendelkező platform törzseket eredményezhet, amelyek alkalmasak lehetnek ipari termelésre.

Curriculum vitae

Roland Sándor Kun was born on the 25th of February, 1992 in Marghita, Romania. After high school, he continued his education with a BSc study in Biochemical Engineering at the University of Debrecen, Hungary. He obtained his BSc diploma in February, 2014. In the same year, he began his MSc studies in Biochemical Engineering at the University of Debrecen, and was involved in fungal research at the Department of Biochemical Engineering, under the supervision of Dr. László Kulcsár and Prof. dr. Levente Karaffa. As part of his Master training, he did a four-month ERASMUS+ internship in the Fungal Physiology group at the Westerdijk Fungal Biodiversity Institute (formerly known as Centraalbureau voor Schimmelcultures (CBS-KNAW)), in Utrecht, the Netherlands, under the supervision of Dr. Tiziano Benocci and Prof. dr. ir. Ronald P. de Vries. Roland obtained his MSc diploma in July 2016. In September of the same year, he started an eleven-month post-graduation ERASMUS+ internship at the Westerdijk Fungal Biodiversity Institute, under the supervision of Dr. Claire Khosravi and Prof. dr. ir. Ronald P. de Vries. In September 2017, Roland started his PhD at the same institute, under the supervision of Dr. Sandra Garrigues and Prof. dr. ir. Ronald P. de Vries. During his PhD he studied diverse applications of the CRISPR/Cas9 system in *Aspergillus niger* and the regulatory network of plant biomass degradation in this species. The results of his work are described in this thesis. After finishing his PhD research in November 2021, he continued his career at the same institute, where he was involved in a short-term project aiming for the production of heterologous proteins in filamentous fungi, under the supervision of Prof. dr. ir. Ronald P. de Vries. Since June 2022, he is working as a postdoctoral researcher at Leiden University, under the supervision of Dr. Arthur F.J. Ram.

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List of publications

First authorships

Kun, R. S., Garrigues, S., Peng, M., Keymanesh, K., Lipzen, A., Ng, V., Tejomurthula, S., Grigoriev, I. V., & de Vries, R. P. (2022). The transcriptional activator ClrB is crucial for the degradation of soybean hulls and guar gum in *Aspergillus niger*. Submitted to *Fungal Genetics and Biology*.

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*Co-first authorship

Co-authorships

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Kun, R. S., Garrigues, S., Di Falco, M., Tsang, A., & de Vries, R. P. Regulation of wheat bran utilization in the industrially relevant filamentous fungus *Aspergillus niger*. The 17th International *Aspergillus* meeting (Asperfest 17) (February 2020), Rome, Italy.

Kun, R. S., & de Vries, R. P. Genetic engineering of the *Aspergillus niger* (hemi-) cellulolytic system using CRISPR/Cas9 technology. Industrial Biotechnology for Lignocellulose Based Processes (October 2019), Gothenburg, Sweden.

Kun, R. S., & de Vries, R. P. Genetic engineering of the *Aspergillus niger* (hemi-)cellulolytic system using CRISPR/Cas9 technology. The 30th Fungal Genetics Conference (March 2019), Pacific Grove, California, USA.

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I think that is all I wanted to say, and now it is time for...

ACCCACGAG GAGAACGAT