Sugar catabolism during growth on plant biomass in *Aspergillus*

Suiker katabolisme van *Aspergillus* gedurende de groei op
plantenbiomassa
(met een samenvatting in het Nederlands)

Le catabolisme des sucres durant la croissance d'*Aspergillus* sur
la biomasse végétale
(avec le résumé en Français)

Proefschrift

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To my father, my mother and my brother

Pour mon père, ma mère et mon frère

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Nederlandse Samenwatting
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List of publications
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Chapter 1

General introduction

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Introduction

Plant biomass is the main renewable material on earth, and is the major starting material for several industrial areas. A growing industrial sector in which plant degrading enzymes are used is the production of alternative fuels, such as bio-ethanol, and biochemicals. The substrate for these conversions is plant material, either from crops specifically grown for this purpose or agricultural waste. Plant polysaccharides can be converted to fermentable sugars by fungal enzymes. The sugars are then fermented to ethanol and other products mainly by yeast (*Saccharomyces cerevisiae*). *Aspergillus* species are organisms of choice for enzyme production for pre-treatment of plant material because it has high levels of protein secretion and it produces a wide range of enzymes for plant polysaccharide degradation [1]. In nature, *Aspergillus* degrades the polysaccharides to obtain monomeric sugars that can serve as a carbon source. Therefore, *Aspergillus* uses a variety of catabolic pathways to efficiently convert all the monomeric components of plant biomass.

In this general introduction, I present an overview of the main carbon catabolic pathways of *Aspergillus niger* and *Aspergillus nidulans* involved in converting the main monomers (D-glucose, D-xylose, L-arabinose, D-galactose, D-mannose, L-rhamnose and D-galacturonic acid) present in plant polysaccharides and their regulation.

Composition of plant biomass

The plant cell wall is composed of primary and the secondary cell wall (Fig. 1) [2]. The primary cell wall consists mainly of complex polysaccharides such as cellulose, hemicellulose, and pectin [3]. It has several functions, e.g. to maintain and determine cell shape, or to control rate and direction of growth. The secondary cell wall is located between the primary cell wall and the plasma membrane (Fig. 1) and contains cellulose and hemicellulose, as well as lignin. Secondary cell walls are formed after the primary cell wall is complete. Inclusion of lignin in the secondary cell wall provides additional protection and rigidity to the cells.

Plant biomass consists mainly of polysaccharides, lignin and proteins (Fig. 1). The composition of plant polysaccharides depends not only on the plant species, but also on the plant tissue, growth conditions (season), and the age at harvesting. The average composition is 40-45% cellulose, 20-30% hemicellulose, and 15-25% lignin. The different plant cell wall polysaccharides interact with each other and with the aromatic polymer lignin to ensure strength and structural form of the plant cell. The different polysaccharides in the plant cell wall contain a variety of monomers (Table 1). Cellulose is a linear polymer of β-1,4-linked D-glucose residues. The cellulose polymers are present as ordered structures, and their main function is to ensure the rigidity of the plant cell wall [4]. The long glucose chains are tightly bundled together into microfibrils by hydrogen bonds to form an insoluble crystalline fibrous material [5]. In addition to this crystalline structure, cellulose microfibrils also contain non-crystalline (amorphous) regions. The ratio of crystalline and non-crystalline cellulose depend on its origin [6].

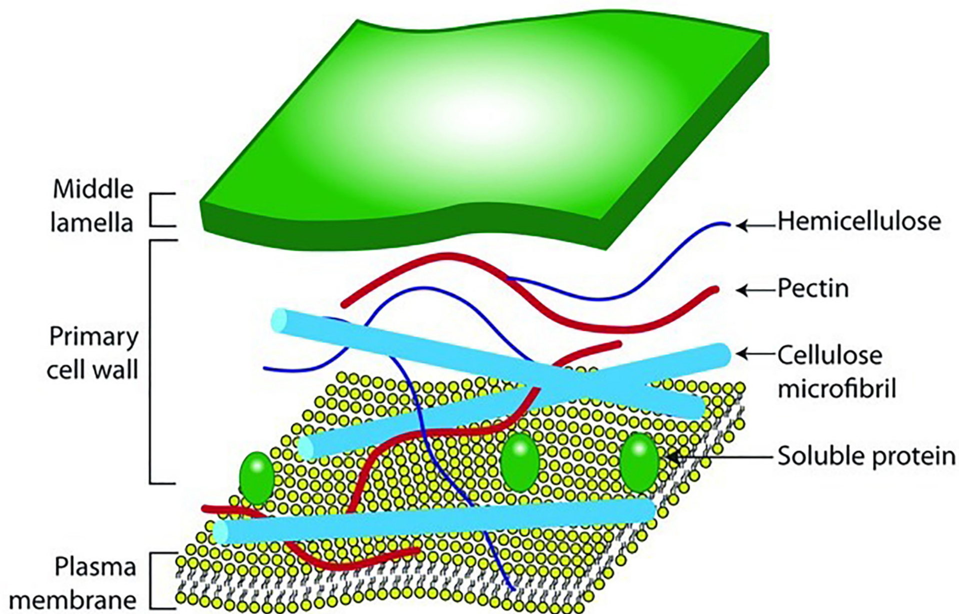

Figure 1 | Plant cell wall structure. Schematic representation of the major structural polysaccharide components of a "typical" primary plant cell wall [7].

Hemicelluloses, the second most abundant polysaccharides in nature, have a heterogeneous composition of various sugar units. Hemicelluloses are usually classified according to the main sugar residues in the backbone of the polymer. The major hemicellulose polymer in cereals and hardwood is xylan. Its consists of a backbone of β-1,4-linked D-xylose residues, which can be acetylated and has mainly α-1,2- or α-1,3-linked L-arabinose (arabinoxylan) and/or α-1,2-linked (4-O-methyl-)D-glucuronic acid (glucuronoxylan) residues attached to the main chain [1]. In addition, it can also contain D-galactose, feruloyl and p-coumaroyl residues [8]. The main xylan present in softwood and cereals is arabinoxylan, whereas hardwood contains mainly glucuronoxylan. A second hemicellulose polymer commonly found in soft- and hardwood is galactoglucomannan. This consists of a backbone of β-1,4-linked D-mannose residues, occasionally interrupted by D-glucose residues with D-galactose side groups (mainly in softwoods). Another hemicellulose, xyloglucan, is present in the cell walls of dicotyledonae and some monocotylodonae (e.g., onion). It consists of β-1,4-linked D-glucose backbone substituted by D-xylose. There are two major types of xyloglucans in plant cell walls: XXGG or XXXG, representing two or three xylose-substituted glucose residues, separated by two or one unsubstituted glucose residue, respectively [9]. Different monosaccharides can be attached to the xylose residues [10]. All hemicelluloses can be acetylated and are cross-linked to cellulose via hydrogen bonds creating a complex and rigid network [11, 12].

Table 1 | Composition of plant polysaccharides. Based on (Kowalczyk et *al.,* 2014) [127].

Polymer type	Polymer	Monomers
Cellulose		D-glucose
Hemicellulose	Xylan	D-xylose
	Glucuronoxylan	D-glucuronic acid, D-xylose
	Arabinoglucuronoxylan	D-xylose, L-arabinose
	Arabinoxylan	D-xylose, L-arabinose
	Galacto(gluco)mannan	D-glucose, D-mannose, D-galactose
	Mannan/galactomannan	D-mannose, D-galactose
	Glucuronomannan	D-mannose, D-glucuronic acid, D-galactose, L-arabinose
	Xyloglucan	D-glucose, D-xylose, D-fructose, D-galactose
	Glucan	D-glucose
	Arabinogalactan	D-galactose, L-arabinose, D-glucuronic acid
Pectin	Homogalacturonan	D-galacturonic acid
	Xylogalacturonan	D-galacturonic acid, D-xylose
	Rhamnogalacturonan I	D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose, ferulic acid, D-glucuronic-acid
	Rhamnogalacturonan II	D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose, L-fucose, D-glucose, D-manno-octulosonic acid (KDO), D-lyxo-heptulosaric acid (DhA), D-xylose, D-apiose, L-acetic acid
Inulin		D-fructose, D-glucose
Starch	Amylose	D-glucose
	Amylopectin	D-glucose
Lignin		Monolignols: p-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol

Pectin is a complex polysaccharide, which is another major component of primary cell wall. It provides rigidity to the cell and plays an important role in porosity, surface charge, pH and ion balance of the cell wall [13]. Pectin contains two different defined regions [1, 14]. The “smooth” regions or homogalacturonan (HGA) consist of a linear chain of α-1,4-linked D-galacturonic acid residues that can be acetylated at O-2 or O-3 or methylated at O-6 [12]. Pectin methyl and acetyl esterases act on this substrate to de-esterify the backbone after which it can be cross-linked by calcium to form a gel, which plays a role in intracellular adhesion [12, 15, 16].

The "hairy" regions contain two different structures, xylogalacturonan (XGA) and rhamnogalacturonan (RG-I). XGA, like HGA, contains an α-1,4-linked D-galacturonic acid backbone that contains β-1,3-linked D-xylose side-groups [17]. RG-I contains an alternating backbone of α-1,4-linked D-galacturonic acid and α-1,2-linked L-rhamnose residues. Long side chains of L-arabinose (arabinan), D-galactose (galactan) or a mixture (arabinogalactan) can be attached to the L-rhamnose residues [18, 19]. The arabinan chains consist of a main chain of α-1,5-linked L-arabinose residues that can be substituted by α-1,3-linked L-arabinose and by feruloyl residues attached terminally to O-2 of the arabinose residues [20]. The galactan side chains contains a main chain of β-1,4-linked D-galactose residues, which can be substituted by feruloyl residues at O-6 [20]. The arabinogalactan side chains can either consist of a backbone of β-1,4-linked galactan substituted with L-arabinose residues or β-1,3-linked galactan, which can be substituted with either β-1,6-linked D-galactose or α-1,3-, α-1,5- and α-1,6-linked L-arabinose residues [20]. Rhamnogalacturonan II (RG-II) is a complex polysaccharide, consisting of approximately thirty monosaccharide units, with a backbone of at least eight D-galacturonic acid residues [21]. The RG-II structure contains five different side chains. These decorations may be mono- or oligosaccharide and can contain several uncommon sugars such as 2-*O*-methyl-L-fucose and 3-deoxy-D-*manno*-2-octulosonic acid [21].

Fungal growth on plant biomass

Fungi are part of one of the major clades of life and form a large and diverse group of eukaryotic organisms. The numbers of fungal species has been estimated at 1.5 million [22]. The major groups which are recognized within fungi are Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota, Glomeromycota, [23]. Fungi can be unicellular (yeast) or multicellular (filamentous fungi) or dimorphic (both yeast and filamentous vegetative growth stage) [4]. Filamentous fungi grow by means of hyphae. These hyphae extend at their tips and branch sub-apically, forming an intricate network. Fungi need to recognize the plant biomass components in order to induce the production of plant biomass degrading enzymes. The polysaccharides present in the plant cell wall are too large to enter the fungal cell and therefore probably cannot act directly as inducers. Fungi likely recognize the presence of complex polymers through smaller monomers derived from them, which acts as signal molecules. Their presence is an indication for the fungus that specific polysaccharides might be present in the environment. When one of these signal molecules is sensed by the fungus, a signaling pathway results in the activation of a transcription factor (TF), which enters the nucleus and triggers the expression of its target genes, encoding CAZymes involved in the plant biomass degradation, as well as the metabolic pathways needed to utilize the available C-source for growth and reproduction [24]. Those signal molecules might be released from complex polysaccharides by the action of enzymes that are constitutively present at low levels [1, 25, 26].

Aspergillus as a plant biomass degrader

The genus *Aspergillus*

The genus *Aspergillus* is assigned to the order Eurotiales in the class of Eurotiomycetes of the phylum Ascomycota [27]. It is cosmopolitan and ubiquitous in nature with over 250 species. Like all filamentous fungi, Aspergilli grow by means of extending hyphae, which branch sub-apically to form a network of hyphae, the mycelium. This mode of growth provides fungi with a large surface area for the uptake of nutrients, and therefore enables them to colonize their substrate in an efficient manner [28]. The genus *Aspergillus* consists mainly of saprobic fungi but some of its members are opportunistic human pathogens*. Aspergillus fumigatus* is the most important of these and is commonly encountered in hospitals as well as other environments [29]. *Aspergillus* species are also known to produce mycotoxins (e.g. *A. flavus* and *A. parasiticus)* [30, 31]. Several *Aspergillus* species are extensively used in industry because of their capability to produce a wide range of enzymes including those that degrade a variety of plant polysaccharides [25]. *Aspergillus nidulans* is an easily manipulated model for genetics and important research organism for studying eukaryotic cell biology [32]. *Aspergillus niger* is widely used for the production of food additives such as citric and gluconic acid, as well as several enzymes [33].The diversity in topics that have been studied in *Aspergillus* has made this the most widely studied genus of filamentous fungi. The number of plant biomass degrading enzymes produced by *Aspergillus* has become more evident since the publication of the genome sequences of many Aspergilli. Comparative analysis of these genomes increases our understanding of the biology of these species and helps us realize their full potential as biomass degraders.

Fungal sugar catabolism

Fungi secrete a large variety of enzymes that act on different polysaccharides or linkages within a polysaccharide [1]. These secreted enzymes can hydrolyse the polysaccharides to pentoses and hexoses, which can be taken up by the fungus and be converted through a variety of metabolic pathways (Fig. 2). The pentoses D-xylose and L-arabinose enter the pentose catabolic pathway (PCP), while D-glucose is a hexose that can be phosphorylated to D-glucose-6P by glucokinase or hexokinase. D-glucose-6P can either enter the PPP or be converted to D-fructose-6P and enter glycolysis. D-fructose that is taken up from the environment is phosphorylated by hexokinase to D-fructose-6P and enters glycolysis. Other hexoses, like D-galactose, D-mannose and L-rhamnose are converted through sugar-specific metabolic pathways forming metabolic intermediates that can enter glycolysis [34].

Catabolism of D-glucose and D-fructose through glycolysis

As mentioned above Aspergilli can degrade the complex structure of the plant cell wall using a large number of extracellular enzymes [1]. One part of this degradation process concerns the release of D-glucose.

It is the major carbon source in the metabolism of most heterotrophic eukaryotes. Although Aspergilli may rarely encounter large amounts of free D-glucose in their natural environment, it is the major component of cellulose and some hemicelluloses. Fungal cellulases, responsible for the release of D-glucose residues, have been classified into three classes: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Endoglucanases hydrolyze cellulose into glucooligosaccharides, while cellobiohydrolases hydrolyze cellulose into cellobiose. These oligosaccharides are further degraded into D-glucose by the action of β-glucosidases. When D-glucose enters the cell it will be phosphorylated to glucose-6-phosphate by either glucokinase (GlkA; EC 2.7.1.2) [35] or hexokinase (HxkA; EC 2.7.1.1) [36]. Glucose-6-phosphate can then either enter the pentose phosphate pathway (see below) or can be converted to fructose-6-phosphate by glucose-6-phosphate isomerase (PgiA; EC 5.3.1.9) [37], SwoM in *A. nidulans* [38] and enter glycolysis (Fig. 3). D-fructose-6-phosphate is converted via phosphofructokinase (PfkA; EC 2.7.1.11) to D-fructose-1,6-diphosphate [39]. It can then be converted to D-glyceraldehyde-3-P by fructose-bisphosphate aldolase (FbaA; EC 4.1.2.13) [40]. Triose-phosphate isomerase (TpiA; EC 5.3.1.1) catalyzes the reversible interconversion of dihydroxyacetone phosphate (DHAP) into D-glyceraldehyde-3-P [41, 42]. 3-Phosphate-glyceraldehyde dehydrogenase (GpdA; EC 1.2.1.12) catalyzes the conversion of glyceraldehyde-3-phosphate to glycerate-1,3-biphosphate [43]. This can then be converted to glycerate-3-phosphate by phosphoglycerate kinase (PgkA; EC 2.7.2.3) [44]. Glycerate-3-phosphate is converted into glycerate-2-phosphate by phosphoglycerate mutase (PgmA; EC. 5.4.2.1) [45], which is then converted into phosphoenol pyruvate (PEP) via an enolase (EnoA; EC. 4.2.1.11) [46] and AcuN in *A. nidulans* [47, 48]. PEP is converted into pyruvate via pyruvate kinase (PkiA; EC. 2.7.1.40) [39] , which is the last step of the glycolysis. The monosaccharide D-fructose is phosphorylated by hexokinase (EC 2.7.1.1) to fructose-6-phosphate, which can enter glycolysis (Fig.3). The genomes of most filamentous fungi such as those of the Aspergilli, contain all necessary glycolytic genes for conversion of D-glucose and D-fructose [45]. Other hexoses derived from plant polysaccharides (such as D-mannose and D-galactose) and C3 metabolites from other catabolic pathways (like D-galacturonic acid, L-rhamnose metabolism) can also be metabolized through glycolysis.

Carbon catabolite repression (CCR) is a regulatory system which prevents wasting energy on the production of extracellular enzymes and metabolic pathways that are not necessary. This energy-saving mechanism is mediated by the CreA repressor protein in *Aspergillus*. This transcription factor is the only one known so far that is conserved throughout the fungal kingdom [49].

Figure 2| Model of the sugar catabolic pathways in fungi.

CreA was first reported in *Aspergillus* by Dowzer and Kelly [50]. In the presence of simple sugars such as D-glucose, the transcription of genes encoding enzymes that degrade other carbon sources is repressed by CreA. However, repression is not only observed in the presence of glucose but also is triggered by other monosaccharides such as D-xylose, D-mannose, and glucuronic acid [51]. In *A. niger*, deletion of CreA leads to derepression of transcription of arabinanolytic [52], xylanolytic and cellulolytic genes [51].

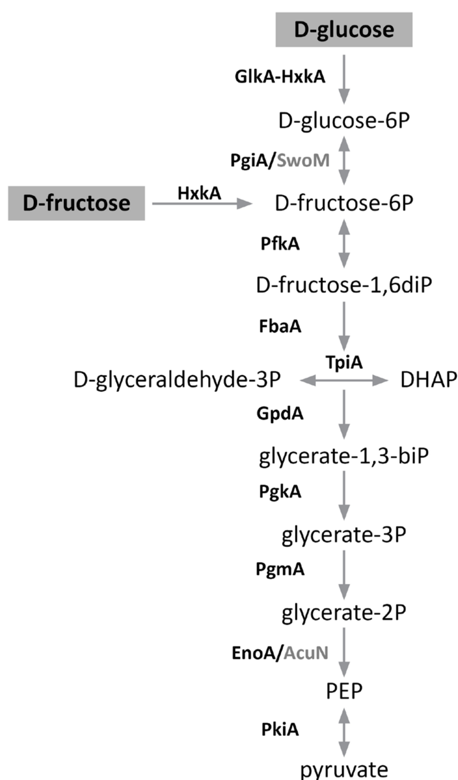

Figure 3 | Glycolysis pathway. HxkA: hexokinase (EC 2.7.1.1); PgiA: phosphoglucoisomerase (EC 5.3.1.9); PfkA/SwoM: glucose-6-phosphate isomerase (EC. 2.7.1.11); FbaA: fructose-1,6-biphosphate (EC 4.1.2.13); TpiA: triose-phosphate isomerase; GpdA: glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); PgkA: phosphoglycerokinase (EC:2.7.2.3); PgmA: phosphoglycerate mutase (EC. 5.4.2.1); EnoA/AcuN: enolase (EC 4.2.1.11); PkiA: pyruvate kinase (EC 2.7.1.40). *A. niger* enzymes are indicated in black and *A. nidulans* enzymes, when different, are indicated in grey.

Pentose phosphate pathway

Glycolysis is connected to the PPP through glucose-6-phosphate, glyceraldehyde-3-phosphate and fructose-6-phosphate. The PPP is a main source of NADPH and of ribose-5-phosphate for synthesis of DNA, RNA and nucleotide cofactors**.** The PPP consists in two phases, the oxidative generation of NADPH and the non-oxidative interconversion of sugars [53]. In the oxidative phase of the PPP, glucose-6-phosphate is oxidized via four enzymatic steps to ribose-5-phosphate and D-xylulose-5-phosphate (Fig. 4).

The first three reactions are catalyzed by glucose-6-phosphate-1-dehydrogenase (GsdA; EC 1.1.1.49) [54], 6-phosphogluconolactonase (PgIA; EC 3.1.1.31) [38] and 6-phosphogluconate dehydrogenase (GndA; E.C 1.1.1.44) [37, 55]. D-ribulose-5-phosphate is converted to D-ribose-5-phosphate by ribose 5-phosphate isomerase A and B (RpiB; EC 5.3.1.6) or to D-xylulose-5-phosphate by ribulose-phosphate 3-epimerase (RpeA; EC 5.1.3.1) [55]. In the non-oxidative phase of the PPP, D-xylulose-5-phosphate and D-ribose-5-phosphate are converted into glyceraldehyde-3-phosphate and D-fructose-6-phosphate by transaldolase (EC 2.2.1.2) and transketolases (EC 2.2.1.1). Regulation of the PCP is under control of the Zn_2Cys_6 transcriptional regulators XlnR and AraR in *A. niger* [56]. A recent study demonstrated that these transcription factors also regulate some PPP genes [56]. Expression of *rpiA* (a ribose-5-phosphate isomerase gene) was reduced in the Δ*araR*Δ*xlnR* strain on L-arabinose and increased in the *xkiA*1 mutant on L-arabinose and D-xylose, indicating direct regulation by XlnR and AraR. The expression profile of *rpiA* in the Δ*araR* and Δ*xlnR* strains on L-arabinose and D-xylose suggests that its expression is influenced by both regulators in a similar manner as it was observed for the PCP genes *xdhA* and *xkiA* [56]. The expression study also suggests that *talB* (a transaldolase gene) is under direct control of AraR and XlnR during growth on L-arabinose and D-xylose, respectively.

Conversion of D-xylose and L-arabinose through the PCP

Due to the heterogeneity of the xylan structure, a wide range of enzymes is required to degrade this polysaccharide. Two classes of enzymes are responsible for release of the xylose residues. Endoxylanases (EC 3.2.1.8) are able to cleave the xylan backbone into smaller oligosaccharides, which can then be degraded further to xylose by β-xylosidases (EC 3.2.1.37) [1]. Several of these enzymes have been identified in *Aspergillus* [57-66]. Another part of the degradation process concerns the release of L-arabinose residues present in hemicelluloses and pectin. Due to the diversity of the structures, the complete release of L-arabinose residues requires the concerted action of four arabinanolytic enzymes: endoarabinanase (EC 3.2.1.99), exoarabinanase, α-L-arabinofuranosidases (EC 3.2.1.55) and arabinoxylan arabinofuranohydrolase [1, 67]. Several α-L-arabinofuranosidases and arabinoxylan arabinofuranohydrolases have been purified from *A. niger* and *A. nidulans* and studied with respect to their activity [61, 68-71]. Two biochemically characterized arabinofuranosidases of *A. niger* are AbfA and AbfB. Arabinoxylan arabinofuranohydrolase (AXH) is specifically involved in arabinoxylan degradation while AbfA and AbfB are more general arabinose-releasing enzymes [72]. Once arabinan is degraded to L-arabinose*,* *Aspergillus* is able to take up and catabolize this pentose using the L-arabinose catabolic pathway (Fig. 4) [73]. In *A. niger,* L-arabinose is reduced by L-arabinose reductase (LarA; EC 1.1.1.21) resulting in L-arabitol (Fig. 4). L-arabitol is then converted to L-xylulose by L-arabitol dehydrogenase (LadA; EC 1.1.1.12) [74]. L-xylulose is reduced to xylitol by L-xylulose reductase (LxrA; EC 1.1.1.10) [75]. Xylitol is then converted to D-xylulose by NAD-dependent xylitol dehydrogenase (XdhA; EC 1.1.1.9).

At this point D-xylose can also enter the pathway after conversion to D-xylulose catalysed by XyrA. As a last step D-xylulose is phosphorylated by D-xylulose kinase (XkiA; EC 2.7.1.17). Xylulose-5-phosphate then enters the PPP (see above) for further conversion. The L-arabitol dehydrogenase encoding gene *(ladA)* has been identified and characterized in three Aspergilli [74, 76, 77], while the genes encoding L-arabinose reductase (*larA*) and L-xylulose reductase *(lxrA*) have recently been characterized in *A. niger* [75, 78]. Genes encoding D-xylose reductase (*xyrA)* [79], xylitol dehydrogenase (*xdhA*) [80] and D-xylulokinase (*xkiA)* [81] have been described for *A. niger*.

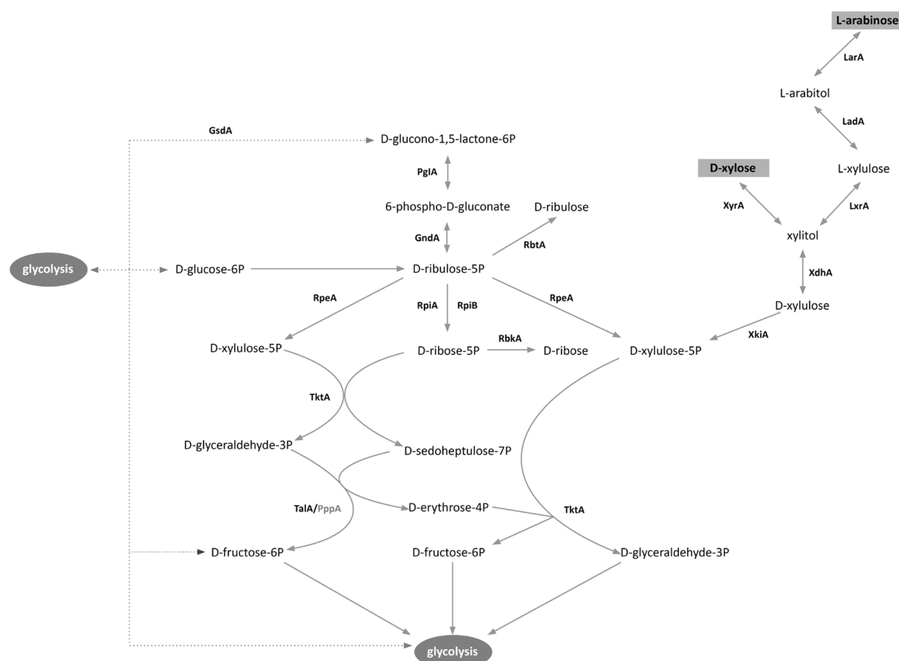

Figure 4 | The fungal pentose catabolic pathway (PCP) and pentose phosphate pathway.
LarA: L-arabinose reductase (EC 1.1.1.21); LadA: L-arabitol dehydrogenase (EC 1.1.1.12); LxrA: L-xylulose reductase (EC 1.1.1.10); XyrA: xylitol dehydrogenase (EC 1.1.1.9); XdhA: xylulokinase (EC 2.7.1.17); XkiA: D-xylulose kinase (EC 2.7.1.17); GsdA: glucose-6-phosphate-1-dehydrogenase (EC 1.1.1.49); PgiA: 6-phosphogluconolactonase (EC 3.1.1.31); GndA: 6-phosphogluconate dehydrogenase (E.C 1.1.1.44); RpeA: D-ribulose-phosphate-3 epimerase (EC 5.1.3.1); TktA: transketolase (EC 2.2.1.1); RbtA: D-ribulokinase; TalA/PppA: transaldolase (EC 2.2.1.2); RpiA-RpiB: ribose 5-phosphate isomerase (EC 5.3.1.6); RbkA: ribokinase (EC 2.7.1.15). *A. niger* enzymes are indicated in black and *A. nidulans* enzymes, when different, are indicated in grey.

Regulation of the PCP is in part dependent on the transcriptional regulator XlnR. XlnR was first identified as a positively acting regulator of the expression of xylanolytic genes in *A. niger* [82] but later studies demonstrated that it also controls cellulolytic genes [83] and xyloglucanolytic genes [84]. Xylan and cellulose are the most abundant polysaccharides in nature, which indicates that XlnR is a critical player in biomass utilization process. Moreover, the activity of XlnR extends beyond polysaccharide degradation as it also controls the first step (D-xylose reductase, *xyrA*) of the PCP. Another transcriptional factor is also acting on the PCP: the L-arabinose related regulator (AraR).

It controls the production of L-arabinose releasing enzymes from pectin and xylan as well as L-arabinose catabolism in *A. niger* and *A. nidulans* [45, 56]. These studies demonstrated that the conversion of D-xylose to xylitol is only controlled by XlnR, while the enzymes converting L-arabinose to xylitol are only controlled by AraR [80]. The conversion of xylitol to D-xylulose-5-phosphate is controlled by both regulators. In *Trichoderma reesei* the whole pathway is regulated by XYR1, an ortholog of *Aspergillus* XlnR that also regulates cellulolytic and hemi-cellulolytic systems. However Stricker et *al.,* [85] suggest that another unknown transcription factor is involved in the control of the pentose phosphate pathway. In *Magnaporte oryzae*, it was demonstrated that XLR1 is not responsible for L-arabinose-related gene expression and no L-arabinose regulator has been identified [86]. Orthologs for XlnR/XYL were found in nearly all filamentous ascomycetes [87], while AraR is restricted to the Eurotiales. This high divergence of regulator and enzyme encoding genes involved in pentose metabolism may be related to fungal evolution in different ecological niches [86].

Catabolism of D-galactose

The degradation of D-galactose residues from plant cell wall polysaccharides requires the action of galactosidases. There are two types of galactosidases: α-galactosidase (EC 3.2.1.22) and β-galactosidase (EC 3.2.1.23) [88]. The α-galactosidases remove D-galactose residues from galacto(gluco)mannan, while β-galactosidases release galactose residues from, xylan, xyloglucan and the galactan side chains of pectins. Microorganisms use several pathways for the catabolism of D-galactose (Fig. 5). The best studied pathway is the Leloir pathway, which is present in prokaryotic and eukaryotic microbes as well as humans [89, 90]. In this pathway, D-galactose is phosphorylated to D-galactose-1-phosphate by galactokinase (GalE; EC 2.7.1.6), which is further converted to D-glucose-1-phosphate by D-galactose-1-phosphate uridyltransferase (GalD; EC 2.7.7.12), UDP-glucose-4-epimerase (GalG; EC 5.1.3.2) and UTP-glucose-1-phosphate uridylyltransferase (GalF; EC 2.7.7.9) [91]. The actual functions of the last two enzymes have not been studied so far. Phosphoglucomutase (PgmB; EC 5.4.2.2) catalyzes the conversion of D-glucose-1-phosphate to D-glucose-6-phosphate, which can be converted into fructose-6-phosphate and enter glycolysis or the pentose phosphate pathway [92].

In *T. reesei*, *A. nidulans* and more recently in *A. niger*, an alternative D-galactose oxido-reductive pathway has been identified (Fig. 5). This pathway converts D-galactose to D-fructose-6-phosphate [93-96]. D-xylose reductase (EC 1.1.1.21) converts D-galactose to D-galactitol in *A. niger* [93], while the aldose reductase that catalyzes this step in *A. nidulans* is unknown [94]. In *A. niger* D-galactitol is converted to L-xylo-3-hexulose by galactitol dehydrogenase (LadB; EC 1.1.1.16) [95]. Its homolog probably also converts galactitol in *A. nidulans* as it is expressed on D-galactose, while *ladA* is mainly expressed on L-arabinose [97]. However, L-sorbose was identified as the product of this reaction in *A. nidulans* [94]. It is still unclear if L-xylo-3-hexulose needs to be converted into L-sorbose before it can be converted to sorbitol. The conversion of L-sorbose into sorbitol is suggested to be catalyzed by L-xylulose reductase (LxrA; EC 1.1.1.10) [98].

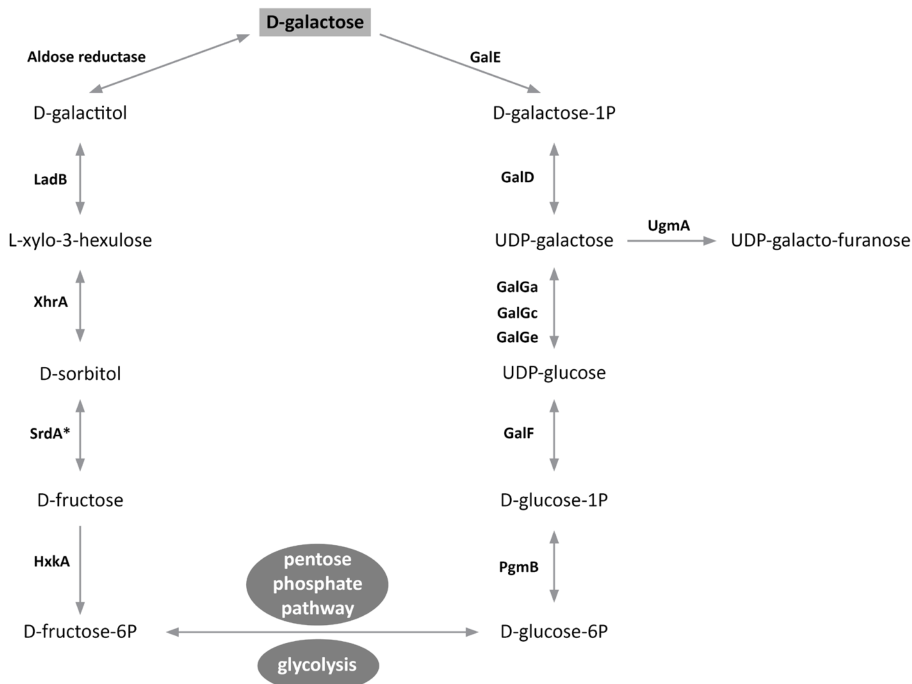

Figure 5 | The metabolic Leloir pathway and the oxido-reductive galactose utilization pathway.
The Leloir pathway (right): GalE: galactokinase (EC 2.7.1.6); GalD: galactose-1-uridyltransferase (EC 2.7.7.12); UgmA: UDP-galactopyranose mutase (EC 5.4.99.9); GalGa-GalGc: UDP-glucose 4-epimerase (EC 5.1.3.2); GalF: UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9); PgmB: phosphoglucomutase (EC 5.4.2.2)
The oxido-reductive pathway (left): HxkA: hexokinase (EC 2.7.1.1); SrdA: sorbitol dehydrogenase (EC 1.1.1.15); XhrA: L-xylo-3-hexulose reductase (EC 1.1.1.-); LadB: galactitol dehydrogenase (EC 1.1.1.16); Aldose reductase (EC 1.1.1.21). *This gene was originally described as *sdhA* in *A. niger* [93]. However, *sdhA* was already previously used for succinate dehydrogenase, so we renamed the sorbitol dehydrogenase gene to *srdA*.

The conversion of sorbitol into D-fructose was described to be catalyzed by sorbitol dehydrogenase (SrdA; EC 1.1.1.14) in *A. niger* [93].
In *A. niger*, a third pathway, the DeLey-Doudoroff pathway, was described for the conversion of D-galactose [99]. This pathway shares characteristics with the Entner-Doudoroff pathway, since the reactions are similar and they result in the same end products: pyruvate and glyceraldehyde. In this pathway, D-galactose is first oxidized to D-galactonic-y-lactonate by D-galactose oxidase (GOase; EC 1.1.3.9) followed by a conversion catalyzed by D-galactono-y-lactonase (EC 3.1.1.25) to D-galactonate. This is then converted to 2-keto-3-deoxy-D-galactonate (KDGal) by D-galactonate dehydratase (EC 4.2.1.6). KDGal is split into glyceraldehyde and pyruvate by KDGal aldolase (EC 4.1.2.14). Only the enzymatic activities of this pathway have been identified so far, while the genes encoding the enzymes are unknown [99]. Two regulators, GalR and GalX, are involved in regulation of the Leloir and D-galactose oxido-reductive pathway genes in *A. nidulans* [91, 100]. GalR was identified by homology to XlnR. It is a member of the Zn_2Cys_6 family of transcriptional regulators. Detailed analysis indicates that GalR and GalX are located next to each other on the *A. nidulans* genome.

While GalX is found in several *Aspergilli*, GalR was reported to be unique to *A. nidulans* [100], but recent availability of additional *Aspergillus* genomes revealed that it is also present in *Aspergillus sydowii* and *Aspergillus versicolor* (R.P. de Vries, unpublished results). A genomic cluster comparison, using the Sybil algorithm [101], showed that *galX* is conserved in most *Aspergilli*, but absent in *Aspergillus terreus, Aspergillus clavatus* and *Neosartorya fischeri* [91].This suggests a different organization of D-galactose catabolism in these related fungi. Microarray analysis showed that GalR regulates the majority of the metabolic genes, whereas GalX regulates the oxido-reductive pathways genes *ladB*, *xrhA* and *sdhA* in *A. nidulans* [100]. Outside *Aspergillus* GalX is also not conserved: comparison between available fungal genome reveals that this regulator is present only in few species, such a *Cladosporium fulvum*, *Nectria haematococca* and *Cryptococcus neoformans* [87]. Homologues of previously described regulators, GalR and GalX in Aspergilli, are not found in *T. reesei*. Recent literature studies revealed no regulators involved in D-galactose catabolism [87]. In *A. nidulans* the D-galactose pathways are pH-dependent: the Leloir pathway is used between pH 4.0 and 6.5 and the oxido-reductive pathway at pH>7 [102]. In *A. niger* it has been hypothesized that the oxido-reductive pathway is the major pathway for D-galactose utilization [95] while in *T. reesei* these two pathways operate simultaneously [96].

Catabolism of D-mannose

The degradation of the mannan backbone needs the synergistic action of β-endomannanases (EC 3.2.1.78), β-mannosidases (EC 3.2.1.25), which are commonly produced by Aspergilli. β-endomannanases hydrolyze mannan into manno-oligosaccharides, while β-mannosidases hydrolyze these oligosaccharides further into mannose [1]. After D-mannose is taken up by the cell, it can be converted to fructose-6-phosphate (Fig. 6). The catabolism of D-mannose is closely linked to glycolysis: D-mannose is phosphorylated by the same hexokinase that phosphorylates D-glucose and D-fructose to D-mannose-6-phosphate. This is converted to fructose-6-phosphate by phosphomannose isomerase (PmiA, EC 5.3.1.8) that enters glycolysis via 6-phosphosfructokinase (PfkA; EC 2.7.1.11) [103]. Mannose-6-phosphate can also be converted to mannose-1-phosphate, catalyzed by phosphomannomutase (PmmA; EC 5.4.2.8) [104, 105]. Mannose-1-phosphate can react with GTP to form (GDP)-mannose and PPi by the action of mannose-1-phosphate guanylyltransferase (MgtA; EC 2.7.7.22).

Recently, the transcriptional regulator of mannanolytic enzymes, named ManR, has been identified in *A. oryzae* [106]. ManR was shown to regulate genes encoding endomannanase, β-mannosidase, α-galactosidase, acetylmannan esterase and β-glucosidase. As expected, disruption of *manR* resulted in a strong decrease in the expression level of genes encoding mannanolytic enzymes such as endo-β-mannanases, β-mannosidases and a putative acetylmannan esterase [106].These indicates that ManR plays a role in galactomannan degradation. ManR is commonly found in Aspergilli, but also in other fungi, such as *Neurospora crassa*, where its ortholog was named Clr-2 [107]. No indications for regulation of mannose catabolism by ManR have been reported.

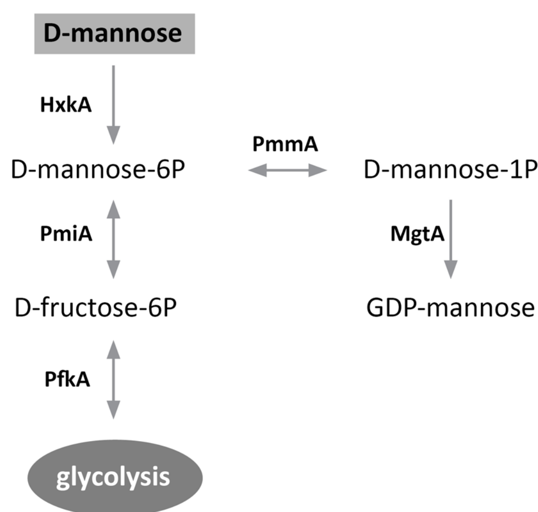

Figure 6 | The fungal D-mannose pathway. HxkA: hexokinase (EC 2.7.1.1); PmiA: phosphomannose isomerase (EC 5.3.1.8); PfkA: 6-phosphofructokinase (EC 2.7.1.11); PmmA: phosphomannomutase (EC 5.4.2.8); MgtA: mannose-1-phosphate guanylyltransferase (EC 2.7.7.22).

Catabolism of L-rhamnose

L-rhamnose is a hexose mainly present in RG-I and II of pectin. Three enzymes are involved in L-rhamnose release. α-Rhamnosidases (EC 3.2.1.40) are able to hydrolyze both L-rhamnose α-1,2- and α-1,6-linkages to L-D-glycosides, and were identified in *A. niger* [108]. Rhamnogalacturonan hydrolases (EC 3.2.1.67) cleave α-galacturonic acid-(1,2)-rhamnose linkages. The corresponding genes were identified in different species, such as *A. niger*, *A. nidulans* and *Botrytis cinerea* [109-111]. Rhamnogalacturonan lyases (EC 4.2.2.-) cleave α-rhamnose-(1,4)-galacturonic acid linkages by β-elimination [112].

There are two distinct pathways known for the catabolism of L-rhamnose: the phosphorylative and the non phosphorylative pathway. The phosphorylative pathway is found only in bacteria such as *Escherichia coli*. L-rhamnose metabolism in fungi was described for the first time in the yeasts *Scheffersomyces stipitis* and *Debaryomyces polymorphus* (Fig. 7) [113] . In this fungal non-phosphorylative pathway, L-rhamnose is first oxidized to L-rhamnono-γ-lactone by L-rhamnose-1-dehydrogenase (LraA; EC 1.1.1.173). This intermediate is converted to L-rhamnonate by L-rhamnono-γ-lactonase (LraB; EC 3.1.1.65) and subsequently to L-2-keto-3-deoxyrhamnonate (L-KDR) by L-rhamnonate dehydratase (LraC; EC 4.2.1.65). This is then cleaved into pyruvate and L-lactaldehyde by L-2-keto-3-deoxyrhamnonate [114]. No LRA4 homologue from *P. stipitis* was found in *A. niger*.

In *A. niger*, a general galacturonic acid-responding regulator appears to activate pectinolytic gene expression [115], but two additional regulators were suggested to control subsets of pectinolytic genes [115]. The first additional regulator responding to the presence of L-arabinose was identified as AraR and the second, RhaR, responding to L-rhamnose was recently described [116].

The *rhaR* gene is expressed in the presence of L-rhamnose and other components of pectin. The disruption of *rhaR* resulted in reduced growth on L-rhamnose indicating that RhaR also controls the expression of the genes of L-rhamnose catabolism in this fungus.
Detailed analysis showed that RhaR affects the expression of the L-rhamnose catabolic genes as well as α-rhamnosidase-encoding genes and also genes encoding other pectinolytic enzymes, such as exorhamnoglacturonases, α-rhamnosidases, rhamnogalacturan acetyl esterases, β-1,4-galactosidases, rhamnogalacturan hydrolase, rhamnogalacturan lyase, feruloyl esterase and pectin acetyl esterase [116], that all act on the pectin substructure I (RG-I). The *rhaR* gene is also present in other *Aspergillus* species, such as *A. oryzae, A. terreus, A. fumigatus* and *N. fischeri* [116]**.**

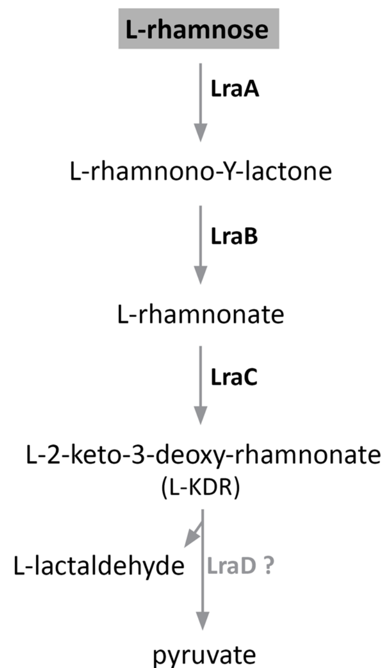

Figure 7 | The fungal L-rhamnose pathway. LraA: L-rhamnose-1-dehydrogenase (EC 1.1.1.173); LraB: L-rhamnono-γ-lactonase (EC 3.1.1.65); LraC: L-rhamnonate dehydratase (EC 4.2.1.90); LraD: L-2-keto-3-deoxyrhamnonate aldolase (EC 4.2.1.-).

Catabolism of D-galacturonic acid

Homogalacturonan, the simplest substructure of pectin is a linear chain of α-(1-4) linked D-galacturonic acid. Four enzymes are required to degrade the homogalacturonan: endo-polygalacturonases (EC 3.2.1.15) and exo-polygalacturonases (EC 3.2.1.67, EC 3.2.1.82), pectin and pectate lyases (EC 4.2.2.10, EC 4.2.2.2) [1].

For bacteria, a catabolic pathway has been described in which five enzymes convert D-galacturonic acid to pyruvate and glyceraldehyde-3-phosphate [117]. Recently a different catabolic pathway was described in *A. niger* [117] (Fig. 8). According to these studies, the enzymes involved in the first step of the pathway differ in these species.

D-galacturonic acid is converted to L-galactonate by a mainly NADPH dependent D-galacturonate reductase (GaaA; EC 1.1.1.365). GaaA is able to use both NADPH and NADH [118]. In the second step L-galactonate is converted to 2-keto-3-deoxy-L-galactonate by L-galactonate dehydratase (GaaB; EC 4.2.1.46) [119, 120]. The third step is catalyzed by a 2-keto-3-deoxy-L-galactonate aldolase (GaaC; EC 4.1.2.54) which splits 2-keto-3-deoxy-L-galactonate in pyruvate and L-glyceraldehyde [120, 121]. Pyruvate can be metabolized by different pathways while L-glyceraldehyde cannot. However, a specific NADPH dependent glycerol dehydrogenase has been reported to be involved in the conversion of L-glyceraldehyde to glycerol (GaaD; EC 1.1.1.cd) [120, 122, 123]. This constitutes the fourth step of the D-galacturonic acid pathway (Fig. 8).

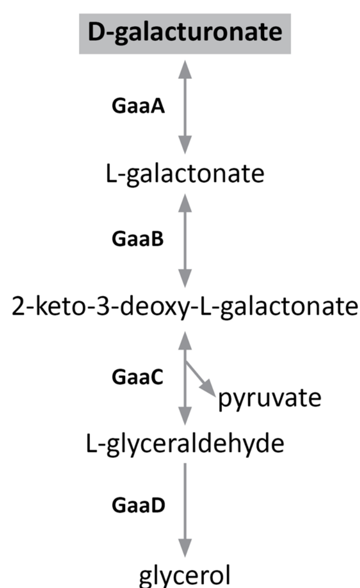

Figure 8 | The fungal D-galacturonic pathway. GaaA: D-galacturonate reductase (EC 1.1.1.365); GaaB: L-galactonate dehydratase (EC 4.2.1.46); GaaC: 2-keto-3-deoxy-L-galactonate aldolase (EC 4.1.2.54.); GaaD: glyceraldehyde reductase (EC 1.1.1.cd).

The complexity of the pectin structure suggests that the regulation of pectinolytic genes may be more complex than regulation of cellulolytic or hemicellulolytic genes. So far, three transcriptional activators (GaaR, RhaR and AraR) and two transcriptional repressors (GaaX, CreA) have been linked to pectin degradation in *A. niger* [111, 116, 124-126]. GaaR controls the expression of genes involved in the degradation of HG, such as exo-polygalacturonases, endo-polygalacturonases, pectin methyl esterases and pectin lyases [124]. In addition to this, recently in *A. niger*, the repressor GaaX was shown to be under control of GaaR [125]. In absence or at low levels of the inducer, GaaX inactivates GaaR.

Aims and outline of the thesis

The aim of this PhD thesis was to study sugar catabolism during growth on plant biomass in *Aspergillus*. Plant biomass is highly complex in structure and consists of many different monomers. If fungi want to use all of these components as carbon source they need a highly varied carbon catabolism that can convert these components into central carbon metabolism. For this, the catabolic pathways for the different sugars all connect to glycolysis. Some of these pathways are highly conserved in fungi, while in other pathways different fungi use different gene sets, adding to the complexity of fungal sugar catabolism. Although much is known about carbon catabolism, recent discoveries of alternate pathways in fungi suggest that there is still much to be learned about catabolic pathways in fungi. An additional level of complexity is the regulation of the pathway genes.
In this thesis, several metabolic pathways involved in the sugar catabolism were studied in detail in *Aspergillus* as well as its regulation (Fig. 9).

Chapter 2 confirmed in *vivo* the function of the first three putative L-rhamnose utilization genes from *A. niger* through gene deletion. This work showed that the inducer of RhaR is beyond L-rhamnonate dehydratase (LraC) and is likely to be the 2-keto-3-L-deoxyrhamnonate. In **Chapter 3** the effect of the deletion of all the D-galacturonic pathway genes (*gaaA, gaaB*, *gaaC* and *gaaD*) on the growth and on the transcription of genes involved in D-galacturonic acid utilization in *A. niger* was analyzed. Higher expression levels of D-galacturonic acid-responsive genes were observed in the Δ*gaaC* mutant compared to the reference strain and other pathway deletion mutants. This indicates that 2-keto-3-deoxy-L-galactonate is the inducer of genes required for GA utilization.

In **Chapter 4**, the effect of *xkiA* and *xlnR* deletion on transcription of genes involved in the degradation of corn stover or soybean hulls was analyzed. A detailed comparative analysis of the CAZy genes involved in the plant biomass degradation is described in both deletion mutants. The results demonstrate the major role of XlnR in the regulation of cellulolytic and hemicellulolytic genes in *A. niger*. In addition, significant differences in the pectinolytic genes were observed in the *xlnR* mutant in soybean hulls compared to corn stover. This showed that the composition of the substrates has an effect on the CAZy gene expression in absence of XlnR.

In **Chapter 5**, the influence of two transcription factors, AraR and XlnR, on the colonization of unprocessed wheat bran and on the enzyme production was analyzed in *A. niger*. The results showed that XlnR is the major regulator required for the colonization of wheat bran.

Even though **Chapter 6** and **Chapter 7** are based on the same dataset, they were separated in two chapters because they respond to different aims. In the **Chapter 6**, we evaluated if it would be possible to create an *A. nidulans* strain that releases but does not metabolize hexoses from plant biomass. The results indicate that the reduced ability to use hexoses as carbon sources created a shift towards the pentose fraction of wheat bran as a major carbon source to support growth.

In **Chapter 7**, the role of the carbon catabolite repressor CreA in the model system *A. nidulans,* during growth on wheat bran was evaluated. The analysis focuses in particular on CAZyme-encoding genes and central carbon metabolism. Our results showed that CreA effects also occur at low monosaccharides levels, in a more natural substrate.

The results are summarized and discussed in **Chapter 8**.

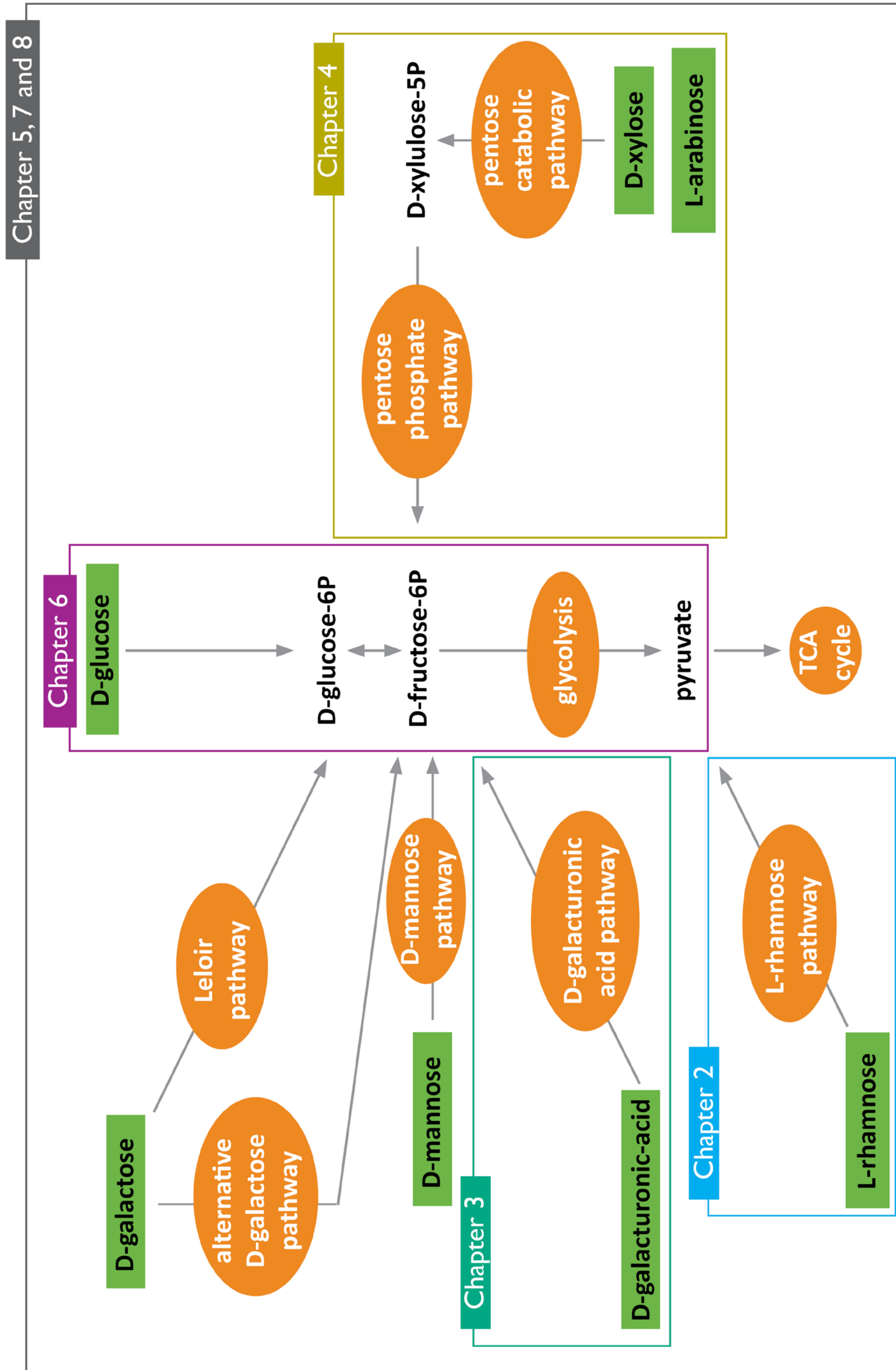

Figure 9 | Schematic outline of the thesis

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Chapter 2

In vivo functional analysis of L-rhamnose metabolic pathway in *Aspergillus niger*: a tool to identify the potential inducer of RhaR

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Abstract

The genes of the non-phosphorylative L-rhamnose catabolic pathway have been identified for several yeast species. In *Scheffersomyces stipitis*, all L-rhamnose pathway genes are organized in a cluster, which is conserved in *Aspergillus niger*, except for the *lra-4* ortholog (*lraD*). The *A. niger* cluster also contains the gene encoding the L-rhamnose responsive transcription factor (RhaR) that has been shown to control the expression of genes involved in L-rhamnose release and catabolism.
In this paper, we confirmed the function of the first three putative L-rhamnose utilisation genes from *A. niger* through gene deletion. We explored the identity of the inducer of the pathway regulator (RhaR) through expression analysis of the deletion mutants grown in transfer experiments to L-rhamnose and L-rhamnonate. Reduced expression of L-rhamnose-induced genes on L-rhamnose in *lraA* and *lraB* deletion strains, but not on L-rhamnonate (the product of LraB), demonstrate that the inducer of the pathway is of L-rhamnonate or a compound downstream of it. Reduced expression of these genes in the *lraC* deletion strain on L-rhamnonate show that it is in fact a downstream product of L-rhamnonate.
This work showed that the inducer of RhaR is beyond L-rhamnonate dehydratase (LraC) and is likely to be the 2-keto-3-L-deoxyrhamnonate.

Introduction

Plant biomass is mainly composed of polysaccharides. In nature, fungi secrete a broad range of polysaccharide degrading enzymes to release monosaccharides, which are then used as nutrients. Pectin is a complex plant cell wall polysaccharide that can be divided into four sub-structures: homogalacturonan (HGA), xylogalacturonan (XGA) and rhamnogalacturonan I and II (RG-I and RG-II) [1]. The backbone of RG-I is composed of alternating L-rhamnose and D-galacturonic acid residues. Long side chains of L-arabinose (arabinan), D-galactose (galactan), or a mixture of L-arabinose and D-galactose residues (arabinogalactan) can be attached to these L-rhamnose residues [2, 3]. *A. niger* is able to efficiently degrade pectin and can utilize L-rhamnose also as the sole carbon source. *A. niger* possesses enzymes that are able to enzymatically release L-rhamnose from RG-I. Endo- and exo-rhamnogalacturonase, α-rhamnosidase, rhamnogalacturonan lyase and rhamnogalacturonan acetyl esterase are all active towards the main chain of RG-I [4, 5]. The L-rhamnose transporter (RhtA) has been characterized recently in *A. niger* [6]. Interestingly, it has been found that *rhtA* is co-localized with the α-L-rhamnosidase gene *rhaB*. The transcriptional profile of *rhtA* and *rhaB* genes during a time-course growth experiment of *A. niger* on L-rhamnose suggests not only that there is a coordinated role in the release and the transport of L-rhamnose during consumption but also that strong activation of these two genes do not require high concentrations of L-rhamnose but only low levels [6]. Two pathways are known for the catabolism of L-rhamnose: a phosphorylative and a non-phosphorylative pathway. The phosphorylative pathway is only found in bacteria such as *Escherichia coli* [7], while the non-phosphorylative pathway is found in bacteria and yeasts [8-12]. In the non-phosphorylative pathway of *Scheffersomyces stipitis*, L-rhamnose is converted to pyruvate and L-lactaldehyde via four metabolic reactions involving a L-rhamnose-1-dehydrogenase (RHA1) that oxidizes L-rhamnose to L-rhamnono-Y-lactone, a L-rhamnono-γ-lactonase (LRA2) that converts L-rhamnono-γ-lactone to L-rhamnonate, a L-rhamnonate dehydratase (LRA3) that converts L-rhamnonate to 2-keto-3-deoxy-L-rhamnonate (L-KDR) and a 2-keto-3-deoxy-L-rhamnonate (L-KDR) aldolase (LRA4) that converts the 2-keto-3-deoxy intermediate to pyruvate and L-lactaldehyde (Fig. 1A). All the genes (RHA1, LRA2, LRA3 and LRA4) have been identified in *S. stipitis* and the corresponding enzymes have been biochemically characterized [8, 13, 14]. The LRA1, LRA2, LRA3, LRA4 genes are organized in a cluster in *S. stipitis* together with a transcription factor with a Zn(II)2Cys6 zinc binuclear cluster domain TRC1 (Fig. 1C). This gene cluster is either fully or partially characterized in other fungal species [13]. In *A. niger*, the homologous genes of RHA1, LRA2, LRA3 designated as *lraA*, *lraB* and *lraC,* as well as the L-rhamnose regulator RhaR [15] are organized in a cluster (Fig. 1C). However, no LRA4 homolog has been found in the cluster in the *A. niger* genome (Fig. 1B). Only one of the *A. niger* L-rhamnose metabolic genes, *lraC*, has been biochemically characterized to encode a L-rhamnonate dehydratase by expressing it in *Saccharomyces cerevisiae* [16].

A double deletion of *lraC* and *lraA* in *A. niger* resulted in a strain that exhibited no L-rhamnose dehydrogenase activity and could not consume or grow on L-rhamnose [17]. It remains unknown which gene function, *lraA* or *lraC*, is actually the cause of the inability of *A. niger* to grow on L-rhamnose. As the *in vivo* function, none of the three individual L-rhamnose pathway genes *(lraA*, *lraB* and *lraC*) has been studied yet in *A. niger.* Our first goal was to perform an *in vivo* analysis of their function by making single gene deletion mutants in *A. niger*. Secondly, we searched and selected *lraD* candidate genes in the *A. niger* genome for making the corresponding gene deletions. And thirdly we made use of the metabolic mutants to identify the inducer of RhaR. In *A. niger*, RhaR has been shown to control expression of genes involved in RG-I degradation, the L-rhamnose transporter gene (*rhtA*) as well as the L-rhamnose catabolic genes (*lraA*, *lraB* and *lraC*) during growth on L-rhamnose [6, 15, 18]. It has been suggested that L-rhamnose or a conversion product thereof could be the inducer of RhaR [15]. Blocking the individual steps in the L-rhamnose pathway would thus allow the identification of the inducer. Either the sugar itself or a metabolic pathway intermediate has been shown to induce other plant biomass related transcriptional regulators, such as L-arabinose/L-arabitol for AraR [19], D-xylose for XlnR [20] and 2-keto-3-deoxy-L-galactonate for GaaR-GaaX activator-repressor module [21]. Therefore, we did not make the deletion mutant of *rhtA* or any other putative L-rhamnose transporter in order to allow always L-rhamnose uptake. Gene deletion and transcriptomic analysis performed in this study showed that *lraA*, *lraB* and *lraC* are required for growth on L-rhamnose and confirmed that RhaR plays an important role in the regulation of L-rhamnose catabolism and the degradation of pectin. The results also indicate that L-rhamnose, L-rhamnono-Y-lactone and L-rhamnonate are not the inducers of RhaR, but that this is a compound located further down in the metabolic pathway.

Materials and Methods

Strains, media and culture conditions

A. niger strain CBS 141257 (Table S1) was used to create the Δ*lraA*, Δ*lraB* and Δ*lraC* strains. The CBS 141257 strain was obtained by transformation of N593.20 with uridine (*pyrG)* from *Aspergillus oryzae* [22]. All *A. niger* strains were grown at 30°C using minimal medium (MM, pH 6) or complete medium (CM, pH 6) [23] with 1,5% of agar. Spores plates contained CM with 2% D-glucose. Plates used in the growth profile contained MM with 25 mM monosaccharides, inoculated with 1000 spores. Liquid cultures of two biological duplicates were inoculated with 10^6spores/ml and incubated in a rotary shaker at 250 rpm and 30°C. Pre-cultures for RNA isolation were grown for 16 hours in 1 L Erlenmeyer flasks containing 250 ml CM with 2% D-fructose. Mycelium was washed with MM and transferred for 2 hours in 250 ml Erlenmeyer flasks containing 50 ml MM supplemented with 25 mM L-rhamnose for RNA-seq. For qPCR analysis, mycelium was transferred for 2 hours in 50 ml Erlenmeyer flaks containing 10 ml MM supplemented with 25 mM carbon source L-rhamnose (Fluka) or L-rhamnonate (Sigma).

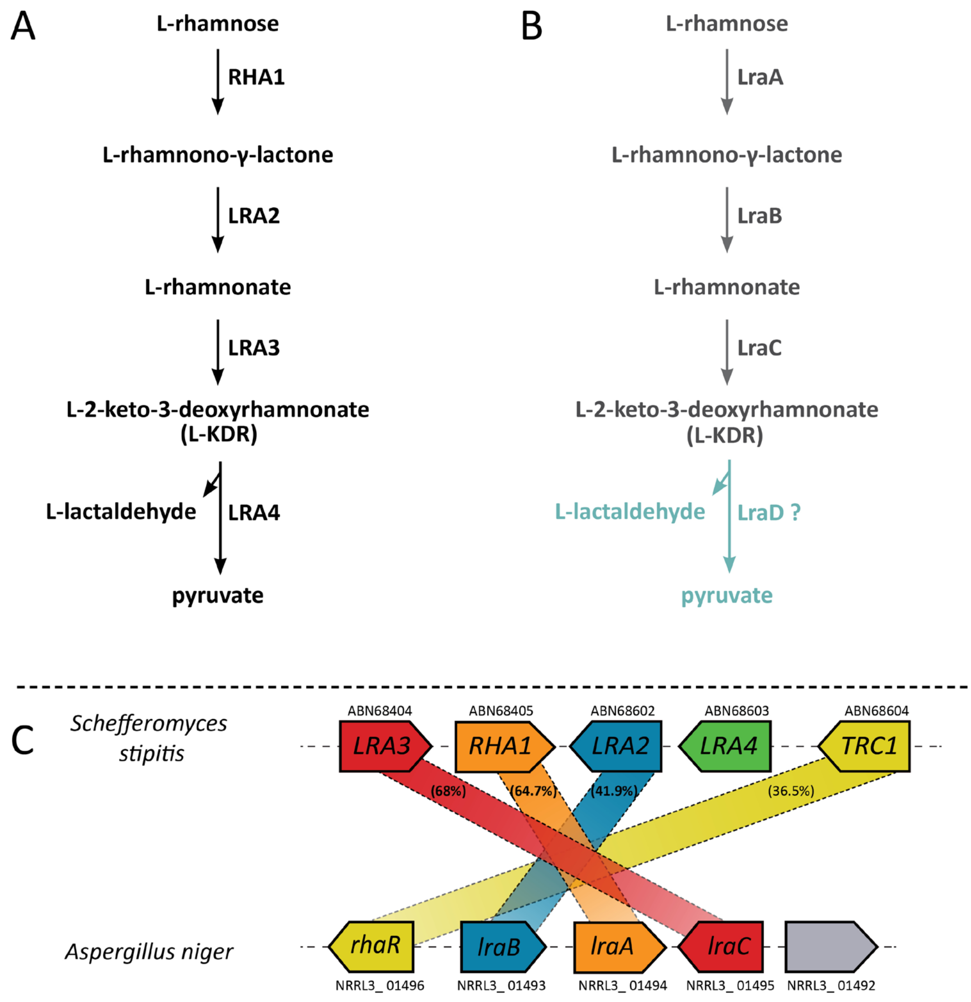

Figure 1 | Schematic representation of the L-rhamnose metabolic pathway in *S. stipitis* (A) and in *A. niger* (B). Orientation of the genes in the gene cluster in *S.stipitis* and *A. niger* (C).
LraA: L-rhamnose-1-dehydrogenase; LraB: L-rhamnono-γ-lactonase; LraC: L-rhamnonate dehydratase; LraD: L-2-keto-3-deoxyrhamnonate aldolase (L-KDR aldolase)

Mycelium was harvested by vacuum filtration, dried between tissue paper and frozen in liquid nitrogen. New strains were deposited at the Westerdijk Fungal Biodiversity Institute with strain numbers indicated in Table S1.

Molecular biology methods and fungal transformation

To construct the deletion cassettes, the upstream and downstream flanks of the genes (*lraA*, *lraB* and *lraC*) were amplified using PCR with gene specific primers (Table S2). The upstream flank reverse primer and downstream flank forward primer carried homologous sequences, overlapping the ends of the *pyrG* selection cassette.

The *pyrG* cassette was amplified from pRV1005 and purified using the Wizard_ SV Gel and PCR Clean-Up Start-Up Kits (Promega). These three fragments were combined in a fusion PCR reaction, to generate the deletion cassette. The fusion PCR mixture contained 1 µl of each PCR product, 12.5 µL GoTaq_ Long PCR Master Mix (Promega), 2 µL of each 10 mM primer in a total volume of 25 µL. The following PCR conditions were used: 2 min at 94°C, 20 s at 94°C, 20 s at 58°C, 5 min at 68°C for 30 cycles and finally an additional 10 min at 68°C. The amplified deletion cassettes were purified using the Wizard_ SV Gel and PCR Clean-Up Start-Up Kits (Promega). Purified DNA (5 µg) of the deletion cassette was used to transform *A. niger*. Protoplast-mediated transformations of *A. niger* were performed as described [24]. DNA was isolated from frozen mycelium, ground with a Tissue Lyser (QIAGEN) using a standard chloroform/phenol extraction. For screening of the *A. niger* transformants, a PCR was performed using genomic DNA of the transformants. Gene-specific sets of primers were used to check the absence or presence of the ORF (Table S2). The resulting strains are listed in Table S1.

RNA extraction, cDNA library preparation and RNA-seq

Total RNA was extracted from mycelium ground in a Tissue Lyser (QIAGEN) using the TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the instructions of the manufacturer. Total RNA samples were purified with the NucleoSpin RNA Clean-up Kit (Macherey-Nagel). Contaminating gDNA was removed by an rDNase solution directly on the silica membrane. RNA integrity and quantity were analyzed on a 1% agarose gel using gel electrophoresis and with the RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA library preparation and sequencing reactions were conducted at BGI Tech Solutions Co., Ltd. (Hong Kong). Illumina library preparation, clustering, and sequencing reagents were used throughout the process following the manufacturer's recommendations (http://illumina.com). mRNA was purified using poly-T oligonucleotide-attached magnetic beads and then fragmented. The first and second strands cDNA were synthesized and end repaired. Adaptors were ligated after adenylation at the 3' end. After gel purification, cDNA templates were enriched by PCR. cDNA libraries were validated using the Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified by qPCR. Single-read samples were sequenced using Illumina HiSeq™ 2000 platform (http://illumina.com). On average 51 bp sequenced reads were constituted, producing approximately a yield of 360 MB raw sequence for each sample.

RNA-seq data analysis and functional annotation

Raw reads were produced from the original image data by base calling. After data filtering, the adaptor sequences, highly 'N' containing reads (> 10% of unknown bases) and low quality reads (more than 50% bases with quality value of < 5%) were removed. After data filtering, on average, ~ 99% clean reads remained in each sample.

Clean reads were then mapped to the genome of *A. niger* NRRL3 (http://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html) using BWA [25, 26]. No more than two mismatches were allowed in the alignment. On average, 70% total mapped reads to the gene was achieved. We refer to *A. niger* gene IDs based on the most up-to-date and accurate annotation of the *A. niger* NRRL3 genome (http://genome.fungalgenomics.ca/). The gene expression level (FPKM) was calculated by using RSEM tool [27]. Genes with expression value higher than 120 were considered highly expressed (approximately top 5%) and differential expression was identified by Student's t-test with a P-value cutoff 0.05. The RNA-seq data have been submitted to Gene Expression Omnibus (GEO) [28] with accession number: GSE99865.

qRT-PCR analysis

cDNA was prepared from total RNA (2.5 µg) using Thermoscript RT (Invitrogen) according to the instructions of the manufacturer. The sequences of all primers for qRT-PCR analysis were designed using the Primer Express 3.0 software (Applied Biosystems). The primers were tested to determine the optimal primer concentrations and efficiency. Combinations of the 50 nM, 300 nM and 900 nM (final concentration) per primer pair were checked, and based on the dissociation curve the optimal primer concentration per primer pair was chosen. The primer sequences of the tested genes and the reference gene are listed in Table S3. qPCR analysis was performed by using the ABI 7500 fast real-time PCR system (Applied Biosystems). The reactions consisted of 2 µl forward and reverse primers at optimal concentration, 20 ng cDNA sample, 10 µl ABI Fast SYBR Master Mix (Applied Biosystems), and water to a final volume of 20 µl. The cycling parameters were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. A dissociation curve was generated to verify that a single product was amplified. Transcript levels were normalized against the histone H2B gene expression and quantified according to the formula 2 –(Ct gene X – Ct H2B) [29]. Two biological and three technical replicates were analyzed.

Results

In vivo effects of deleting the L-rhamnose metabolic genes

The candidate genes for the first three enzymatic reactions of the L-rhamnose pathway are homologs of the characterized genes of *S. stipitis* [15]. The single deletions in NRRL3_08837 (Δ*lraA*), NRRL3_01493 (Δ*lraB)* and NRRL3_10522 (Δ*lraC)* resulted in strains that are unable to grow on L-rhamnose (Fig. 2) compared to the reference strain. A similar growth phenotype was observed on L-rhamnose for the Δ*rhaR* strain (Fig. 2). The small colonies observed for the metabolic deletion mutants on L-rhamnose is background growth since the plates looked similar to our control agar plates without any carbon source added (Fig. 2). Growth of the metabolic deletion mutants on D-glucose was similar to the reference (Fig. 2).

This result indicates that all three genes of the pathway are essential for the conversion of L-rhamnose. Growth of Δ*lraA* and Δ*lraB* was reduced on L-rhamnonate, while no growth was observed in the Δ*rhaR* and Δ*lraC* on L-rhamnonate (Fig. 2).

Previously, it was already shown by performing a bidirectional BLAST analysis that the closest homolog of *S. stipitis* LRA4 is NRRL_08779 [15]. However, this gene is not found in the *A. niger* L-rhamnose cluster (Fig. 1C), it is not induced on L-rhamnose compared to D-glucose and its expression is not affected in the Δ*rhaR* mutant on L-rhamnose compared to the reference strain (Table 4). In addition, it has a very low expression level in all growth conditions tested. In order to search for candidate genes for the L-KDR aldolase (*lraD*), we analyzed the *A. niger* genome for genes containing any of the InterPro and PFAM domains (Table S4), similar domains to those found in *S. stipitis* LRA4 [13].

Next, we compared the expression between L-rhamnose and D-glucose and we ranked all candidate genes by fold-change and selected three genes (NRRL3_03899, NRRL3_05649, NRRL3_06731). The first candidate was 39-fold up-regulated, the second one was 24-fold up-regulated and the third one was 3-fold up regulated on L-rhamnose in comparison to D-glucose (Table S4). The other genes were either not induced on L-rhamnose compared to D-glucose or not expressed at all. These three candidate genes for *lraD* were deleted in the *A. niger* CBS 141257. Growth phenotypic analysis of the mutants (Δ*lraD1*, Δ*lraD2,* Δ*lraD3*) was performed and growth on L-rhamnose was compared to the reference strain. Deletion of the genes did not affect growth on L-rhamnose and L-rhamnonate (Fig. 2), suggesting that these genes are not the functional homologs of *S. stipitis* LRA4.

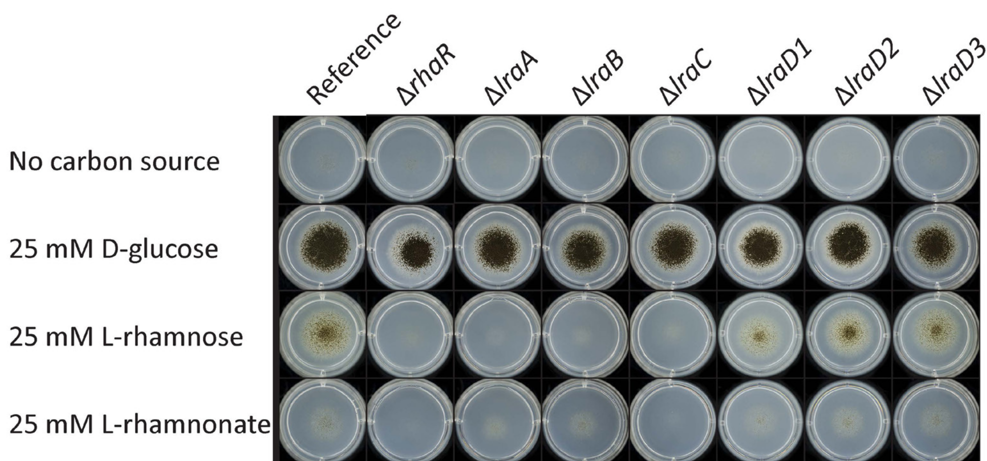

Figure 2 | Growth profile on L-rhamnose, L-rhamnonate and D-glucose of the reference strain and the deletion mutants. The reference, Δ*lraA*, Δ*lraD1*, Δ*lraD2* and Δ*lraD3* were grown on MM with 25 mM L-rhamnose, 25 mM L-rhamnonate and 25 mM D-glucose for 4 days at 30°C. Spore inoculations were done with 1000 spores.

L-KDR is most likely the inducer of the pathway specific regulator RhaR

RhaR was previously identified as an L-rhamnose-responsive transcription factor gene with a role in induction of genes involved in L-rhamnose transport, catabolism and in RG-I degradation [6, 15]. To test the effect of the three metabolic gene deletions on the induction of L-rhamnose responsive genes, RNA-seq analysis was performed. The reference strain and the KO strains Δ*lraA*, Δ*lraB*, Δ*lraC* and Δ*rhaR* were pre-grown in liquid cultures containing MM with fructose and then transferred to MM with L-rhamnose. RNA-seq analysis showed that the expression levels of *lraA*, *lraB* and *lraC* were close to 0, in Δ*lraA*, Δ*lraB* and Δ*lraC*, respectively (Fig. 3) confirming the deletion of these genes. When *lraA, lraB* or *lraC* are deleted*,* the expression level of the other genes of the L-rhamnose pathway is reduced (Fig. 3). A high expression level was observed for *rhaR* in the reference, which was reduced to almost zero in Δ*lraC*, but only a small reduction in its expression was visible in Δ*lraA* and Δ*lraB* (Fig. 3). In the Δ*rhaR* mutant, expression of *lraA*, *lraB* and *lraC* is strongly reduced, confirming that these genes are under control of RhaR, as previously reported [15]. In the Δ*lraA* and Δ*lraB* mutant, the expression level of the L-rhamnose transporter gene (*rhtA*) is strongly reduced (Fig. 3; Table S5). However, *rhtA* was not expressed in the Δ*lraC* and Δ*rhaR* mutants (Fig.3). Only one of the other candidate L-rhamnose transporter genes described previously [6] showed a similar gene expression profile in the reference strain and the metabolic mutants (NRRL3_02828).

In summary, these results show that in the Δ*lraA*, Δ*lraB* or Δ*lraC* mutant, the transcript level of the other metabolic pathway genes and the transporter gene is reduced. It is most likely that this reduction in gene expression is due to lack of inducer formation and that accumulation of L-rhamnose, L-rhamnono-y-lactone and L-rhamnonate does not result in hyper-induction of RhaR-regulated genes as was seen previously for the *A. niger* Δ*gaaC* mutant during growth on D-galacturonic acid [21]. These results also demonstrate that L-rhamnose, L-rhamnono-γ-lactone and L-rhamnonate are not the inducers of RhaR, and we therefore hypothesized that the inducer is formed further down in the L-rhamnose catabolic pathway and it therefore might be L-KDR.

To strengthen this hypothesis, we checked the expression of six rhamnose-induced genes after 2 hours of transfer of the metabolic mutants to L-rhamnonate as well. Fructose pre-grown mycelium of the reference, Δ*rhaR*, Δ*lraA*, Δ*lraB* and Δ*lraC* strains were transferred to MM supplemented with either L-rhamnose or L-rhamnonate. qPCR analysis showed reduced expression levels for *lraA*, *lraB*, *lraC*, *rhaR*, rhamnogalacturonan lyase (*rglB)*, exo-rhamnogalacturonan hydrolase *(rgxA)* and rhamnogalacturonan acetylesterase *(rgaeA)* in the *lraA* and *lraC* mutants compared to the reference, after 2 hours of culture in L-rhamnose, confirming our RNA-seq data results (Fig. 4A-G). Except for *rhaR* and *rgaeA*, the expression level of the genes is higher in Δ*lraB* compared to the reference strain (Fig. 4D and 4G). Interestingly, all six genes were induced during 2 hours of growth of the reference strain on L-rhamnonate (Fig.4H-N). Transcript levels were similar to those observed for the reference strain on L-rhamnose (Fig.4A-G).

Deletion of either *lraA* or *lraB* did not affect the expression of the other metabolic genes, the *rhaR* gene and the three RG-I specific genes. However, strongly reduced expression levels of *lraA*, *lraB*, *rhaR*, *rglB*, *rgxA* and *rgaeA* were observed in the Δ*lraC* mutant upon the 2 hours transfer to L-rhamnonate compared to the reference strain (Fig. 4H-N).

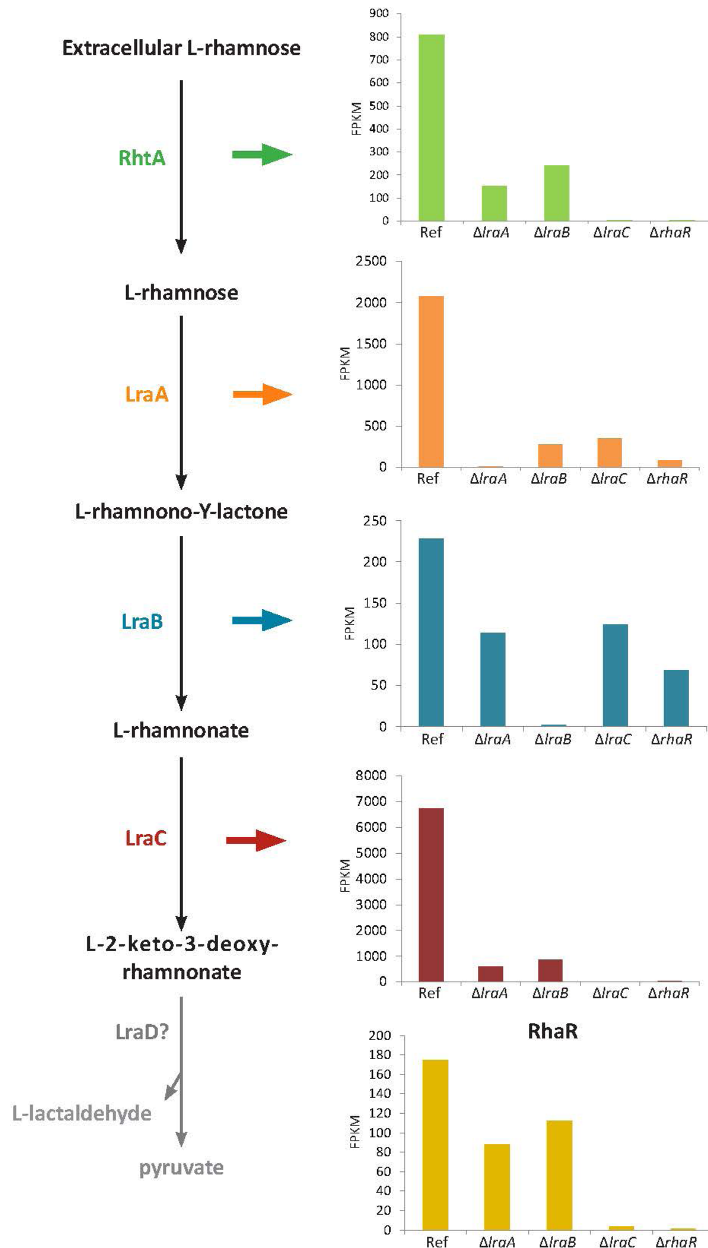

Figure 3 | Schematic representation of the expression of *rhtA*, *lraA, lraB* and *lraC* in the L-rhamnose pathway and *rhaR* in *A. niger* Δ*lraA*, Δ*lraB*, Δ*lraC* and Δ*rhaR* strains after 2 hours of transfer from fructose to liquid medium containing 25 mM of L-rhamnose. Gene expression values are presented in FPKM.

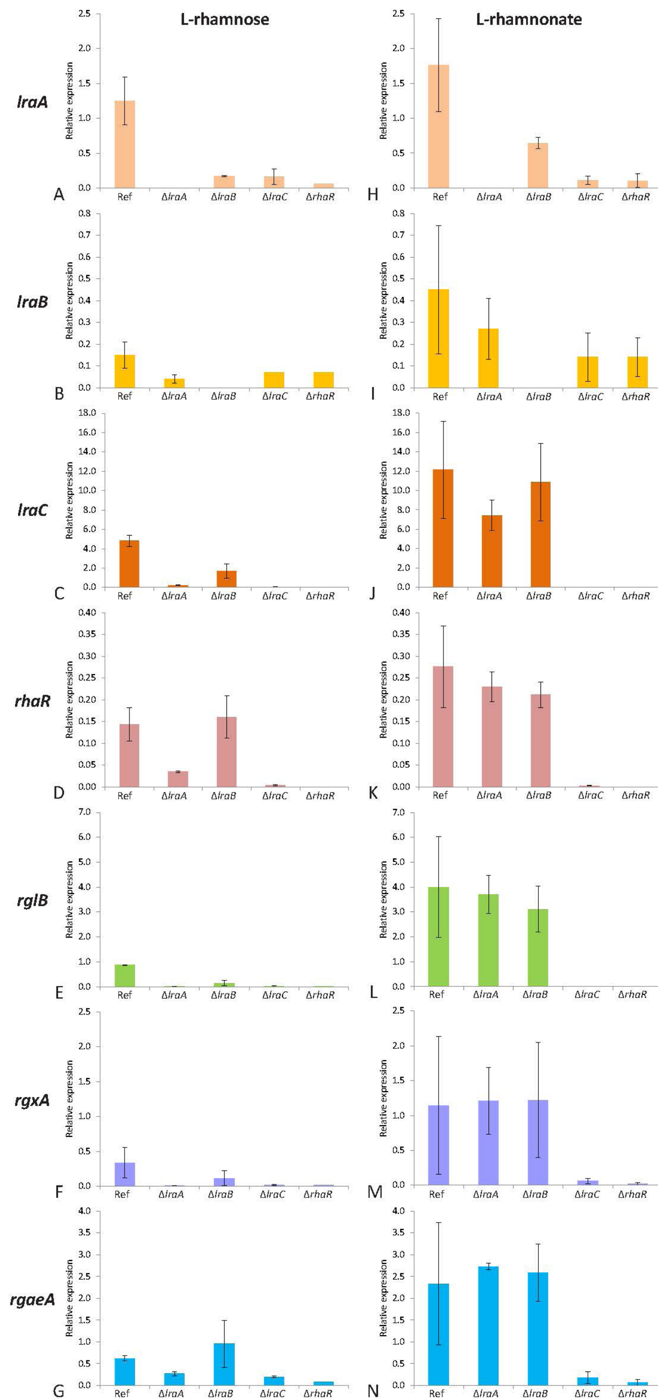

Figure 4 | Expression levels of *lraA* (A;H), *lraB* (B;I), *lraC* (C;J), *rhaR* (D;K), *rglB* (E;L), *rgxA* (F;M)and *rgaeA* (G;N) in *A. niger* Δ*lraA*, Δ*lraB*, Δ*lraC* and Δ*rhaR* mutants . The expression was measured in the reference and single mutant strains of *A. niger* after a transfer for 2 hours on L-rhamnose (A-G) and L-rhamnonate (H-N). The columns represent the mean and error bars represent standard deviation between biological replicates.

CAZy genes involved in degradation of the RG-I backbone were down-regulated in the L-rhamnose pathway mutants

The expression of pectinolytic genes was compared between the L-rhamnose pathway mutants and the reference strain after a transfer for 2 hours from fructose to liquid medium containing 25 mM of L-rhamnose to study the transcript profiles of all rhamnose-induced genes in detail. Pectinolytic genes were divided into subclasses, depending on where they act on the pectin backbone: homogalacturonan (HGA), xylogalacturonan (XGA), rhamnogalacturonan-I (RG-I) and side chains (SC) (Table S6).

Most of the genes involved in the degradation of the RG-I backbone were down-regulated in the *lraA*, *lraB*, *lraC* and *rhaR* deletion mutants compared to the reference strain: two genes encoding GH28 exo-rhamnogalacturonases (*rgxA:* NRRL3_02832; *rgxB*: NRRL3_08631), four putative GH78 α-rhamnosidases (NRRL3_02162; NRRL3_06304; NRRL3_03279; NRRL3_07520), one gene encoding a GH105 unsaturated rhamnogalacturonan hydrolase (*urhgA*; NRRL3_00839), one gene encoding a PL4 rhamnogalacturonan lyase (*rglB*; NRRL3_10115) and two genes encoding CE12 rhamnogalacturonan acetyl esterases (*rgaeA*; NRRL3_00169 and *rgaeB*; NRRL3_07501) (Table 1 and Table S6). Two pectinolytic genes involved in the degradation of HGA and XGA, one PL1 pectin lyase (*pelF*; NRRL3_04153) and one CE12 pectin acetyl esterase (*paeA*; NRRL3_06053), showed a strongly reduced expression level on L-rhamnose in all metabolic deletion strains and in Δ*rhaR* compared to the reference strain (Table S6). Two genes involved in the degradation of the pectin side chains, one GH3 β-xylosidase gene (*xlnD*; NRRL3_02451) and one GH35 β-1,4-galactosidase gene (*lacC*; NRRL3_11738) showed a similar transcript profile.

Two GH43 endoarabinases showed different expression profiles, *abnA* (NRRL3_00092) was highly expressed in Δ*lraC* and (*abnC*; NRRL3_05407) was up-regulated in Δ*lraA,* Δ*lraB* and Δ*lraC* compared to the reference.

Discussion

In this study, we constructed single deletion mutants to confirm *in vivo* the function of the first three genes of the L-rhamnose catabolic pathway in *A. niger* (Δ*lraA*, Δ*lraB* and Δ*lraC*). These strains, together with the Δ*rhaR* mutant, were also used to investigate L-rhamnose-induced transcriptional up-regulation by RhaR in *A. niger* to elucidate the inducer molecule. Comparative growth analysis of the three L-rhamnose catabolic mutants showed that individual deletion of *lraA*, *lraB* and *lraC* results in an inability to use L-rhamnose as a sole carbon source. This indicates that there are no other enzymes capable of replacing the function of LraA, LraB and LraC, at least at a level that can rescue the growth phenotype on L-rhamnose. These results are in line with a previous study in which a double deletion mutant of *lraA* and *lraC* in *A. niger* was unable to grow on L-rhamnose [17, 27].

The second aim of our study was to search for *lraD* candidate genes in the *A. niger* genome and identify this function by gene deletion. L-rhamnose catabolism genes (RHA1, LRA2, LRA3 and LRA4) have been previously found in a chromosomal gene cluster in *S. stipitis* [8, 13]. In *A. niger*, the orthologs of the *S. stipitis* RHA1, LRA2 and LRA3 genes ,the *lraA, lraB* and *lraC* genes, are clustered with *rhaR* on chromosome II, but the cluster does not contain an LRA4 homolog (*lraD)* [15].
In this study we selected three candidate genes for *lraD* that were specifically up-regulated in L-rhamnose and which all have similar PFAM and InterPro domains to those found in LRA4 of *S. stipitis.* However, deletion of these genes did not reduce growth on L-rhamnose, suggesting that neither of them encodes an L-KDR specific aldolase with a key role in L-rhamnose metabolism. Because the five remaining genes in the *A. niger* genome with a dihydrodipicolinate synthetase family domain (PF00701) were not induced on L-rhamnose or not expressed in any condition (Table S4), it is very unlikely that these genes are involved in L-rhamnose metabolism in *A. niger*. A possibility is that the real *lraD* gene of *A. niger* belongs to a different aldolase family than the LRA4 of *S. stipitis*. Also, we cannot exclude that an alternative enzyme could convert L-KDR in the L-rhamnose pathway of *A. niger*. In *Sphingomonas sp*, a gene cluster consisting of LRA1-3, LRA5 and LRA6 has been found. LRA5 and LRA6 were assigned as new enzymes, L-KDR-4-dehydrogenase (KDRDH) and 2,4-diketo-3-deoxy-L-rhamnonate hydrolase (DDRH), respectively [11]. LRA5 (KDRDH) was identified as an NAD+ specific enzyme belonging to the short-chain dehydrogenase/reductase (SDR) superfamily [11] and has been shown to convert L-KDR to 2,4-diketo-3-deoxy-L-rhamnonate. Interestingly, another KDRDH belonging to the medium chain dehydrogenase reductase (MDR) superfamily has been biochemically characterized in *Sulfobacillus thermosulfidooxidans* and was found to catalyze the same metabolic reaction [10]. We analysed and compared the PFAM domains in the protein sequences of LRA5 of both species. The KDRDH from *Sphingomonas sp*. contains an enoyl-(Acyl carrier protein) reductase domain (PF13561) and the KDRDH from the acidophile *S. thermosulfidooxidans* contains an alcohol dehydrogenase GroES-like domain (PF08240) and a zinc-binding dehydrogenase domain (PF00107). In *A. niger*, a putative alcohol dehydrogenase (NRRL3_01492) is present in the *lraA-C* cluster (Fig.1C), which is 33-fold up-regulated in the micro-array data of *A. niger* wild-type in L-rhamnose compared to D-glucose [15]. This putative alcohol dehydrogenase contained the same PFAM domains as KDRDH from *Sulfobacillus thermosulfidooxidans.* Interestingly, this putative alcohol dehydrogenase is well conserved within the genomes of the other Aspergilli [15]. This putative alcohol dehydrogenase might be a likely candidate in *A. niger*, to convert L-KDR into 2,4-diketo-3-deoxy-L-rhamnonate.
The third aim was to study the transcript profiles of the L-rhamnose-induced genes in the metabolic deletion mutants to identify the inducer of RhaR in *A. niger.* In our RNA-seq analysis, genes involved in the L-rhamnose catabolism and in RG-I degradation were significantly lower expressed in Δ*lraA*, Δ*lraB* and Δ*lraC* mutants compared to the reference strain.

Our results revealed that 12 of the 23 RG-I related pectinolytic genes were >1.5 fold down-regulated in all the deletion strains compared to the reference strain on L-rhamnose. In the D-galacturonic acid pathway in *A. niger*, deletion of *gaaA* and *gaaB* resulted in reduced expression profiles of pectinolytic genes on D-galacturonic-acid compared to the reference [21], while deletion of *gaaC* resulted in up-regulation of these genes whereas no difference was observed for the deletion of *gaaD*. This study also demonstrated that the inducer of the galacturonic acid degradation route is 2-keto-3-deoxy-L-galactonate, which is the substrate for GaaC, indicating that accumulation of an intermediate due to deletion of a pathway gene allows the identification of the inducer of the pathway. Since the expression of L-rhamnose-responsive genes was reduced in the strains in which *lraA*, *lraB* or *lraC* were deleted, this indicates that none of these deletions results in accumulation of the inducer of the pathway regulator (RhaR). The expression did not reduce to zero for all the known rhamnose catabolic genes in all the strains, suggesting that there is either a basal non-regulated expression of these genes or alternatively that they are also under control of other regulatory systems.

Based on an alignment between TRC1 in *S. stipitis* and RhaR in *A. niger*, regions of the DNA Binding domain are conserved. This correlates with the phylogenetic analysis performed in Koivistoinen et *al.,* 2012 [13]. Therefor RhaR appears to be an orthologue of TRC1. Also, since the transcription factor RhaR is conserved within the genomes of the other Aspergilli, and is also conserved in fungal species, we postulate that the product of the LraC reaction, L-KDR, is the inducer of the system. In *S. stipitis* another transcription factor than TRC1 has been found in the L-rhamnose cluster, FST14. This transcription factor was more up-regulated than TRC1 on L-rhamnose and it might be co-regulating the pectinolytic genes together with TRC1 [13]. This could also be the case in *A. niger* as previously suggested [15], even though we could not find an orthologue for this regulator in *A. niger*.

The L-rhamnose transporter encoding gene and the metabolic genes are L-rhamnose-induced and in the qPCR and RNA-seq analysis they have a similar gene expression profile in the metabolic and *rhaR* deletion mutants. These results and those of Sloothaak et *al.,* 2016 [6] indicate that L-rhamnose is predominantly transported via the RhtA transporter and then converted through the L-rhamnose metabolic pathway.

Previously it was shown that only a small concentration of L-rhamnose is enough to induce the system [6], as also observed for the D-xylose regulatory system in *A. niger* [30]. The very low expression levels of *lraA*, *lraB*, *rhaR*, *rglB*, *rgxA*, *rgaeA* obtained in Δ*lraC* in the presence of L-rhamnonate showed that the third step is indeed necessary to generate the inducer. In the Δ*lraA* and Δ*lraB* strains the inability to generate the inducer from L-rhamnose can be overcome by supplying L-rhamnonate instead, which is the product of LraB. This then also explains why Δ*lraA* and Δ*lraB* can still grow on L-rhamnonate, while Δ*lraC* cannot. RNA-seq combined with the qPCR results of the L-rhamnose metabolic mutants demonstrated that L-rhamnose, L-rhamnono-γ-lactone and L-rhamnonate are not the inducers of RhaR.

Interestingly and in contrast to transfer to L-rhamnose, upon transfer to L-rhamnonate the reference, Δ*lraA* and Δ*lraB* strains showed similar expression levels in all the genes tested. L-rhamnonate is unlikely to use the L-rhamnose transporter RhtA, due to its different chemical nature. The higher levels of expression observed in the reference, Δ*lraA* and Δ*lraB* strains suggest that by using L-rhamnonate as carbon source which avoids the first 2 steps in the L-rhamnose metabolic pathway, its metabolism still leads to induction of the L-rhamnose pathway. But induction requires a functional LraC since induction is severely affected in the *lraC* deletion strain
Because the gene or genes involved in the conversion of L-KDR remain unknown in *A. niger*, studying metabolic gene deletion mutants further downstream of the LraC step is required to unambiguously establish the identity of the inducer. At this point in time we do not know whether L-KDR is converted by a yet unknown aldolase into L-lactaldehyde and pyruvate or, alternatively by LRA5 and LRA6 homologs to L-lactate and pyruvate. The putative alcohol dehydrogenase present in the cluster, which on the basis of the PFAM domains found and its rhamnose-responsive expression, makes it more likely that the L-rhamnonate degradation pathway involves formation of 2,4-diketo-3-deoxy-L-rhamnonate from L-KDR. The final C3 metabolites formed (pyruvate and L-lactaldehyde or L-lactate) via these two non-phoshorylating pathways have to be further metabolized via gluconeogenesis. These metabolites are already part of central metabolism and are not expected to play any role in the induction of the rhamnose pathway itself. Lactaldehyde dehydrogenase is involved in the conversion of L-lactaldehyde to L-lactate. Several putative lactaldehyde dehydrogenases (AldA) based on a characterized AldA in *Escherichia coli* were found in *A. niger* [31]. The first putative gene (NRRL3_11302) shares 33.3% identity with AldA from *E. coli* and it is significantly up-regulated in the reference strain compared to all the deletion mutants. Also, one putative lactate dehydrogenase (LDH; NRRL3_07593) that catalyzes the reaction from lactate to pyruvate, shares 37.4% identity with a characterized LDH from *Rhizopus oryzae* [32]. RNA-seq results showed that this putative gene is not induced after 2 hours of transfer. This potentially leaves us with two options for the identity of the inducer, viz. L-KDR or 2,4-diketo-3-deoxy-L-rhamnonate, and further work will be required to discriminate between these two options.

In conclusion, this study confirmed the function of *lraA*, *lraB* and *lraC* in the *A. niger* L-rhamnose catabolic pathway *in vivo*. Our search for candidate genes for the L-KDR aldolase (*lraD*) resulted in selection and deletion of three genes, but none of them showed to have a role in the L-rhamnose pathway. Based on further conserved domain searches with previously characterized L-rhamnose metabolic genes, we suggest that L-KDR may be converted via an unknown aldolase or via an unknown KDRDH.

The qPCR-analysis showed that L-rhamnonate catabolism requires LraC and RhaR but not L-rhamnose as an inducer. LraA and LraB are not required for the conversion of L-rhamnonate into L-KDR. This also strongly suggest that the L-rhamnose pathway intermediate L-KDR is the real inducer of RhaR in *A. niger*.

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Supplementary material

Table S1 | Strains used in this study.

Strain	CBS accession number	Genotype	Description	Reference
FP-1132.1 (Reference)	CBS 141257	*cspA1, pyrG⁻::AOpyrG, kusA::amdS*	Restored pyrG in N539.20	[22]
Δ*lraA*	CBS 141252	*cspA1, pyrG⁻, kusA::amdS, lraA::AOpyrG*	Δ*lraA* in N539.20	This study
Δ*lraB*	CBS 141253	*cspA1, pyrG⁻, kusA::amdS, lraB::AOpyrG*	Δ*lraB* in N539.20	This study
Δ*lraC*	CBS 141254	*cspA1, pyrG⁻, kusA::amdS, lraC::AOpyrG*	Δ*lraC* in N539.20	This study
Δ*rhaR*	CBS 137440	*cspA, ΔkusA::amdS⁺,pyrG⁻ ,ΔrhaR ::pyrG⁺*	Δ*rhaR* in N539.20	[15]

Table S2 | Primers used in this study. Overlapping sequences for fusion PCR are written in bold. **Available upon request from the author.**

Table S3 | Primers used in this study to generate the gene fragments for qRT-PCR analysis. **Available upon request from the author.**

Table S4 | Identification of candidate LraD genes in *A. niger.* The data are organized according to the fold changes (highest to smallest). The fold change is the difference in expression between the reference on rhamnose over glucose. p-value <0.05 (cells marked yellow). In red are indicated the three LraD candidates selected. **(See below)**

Table S5 | Putative transporter genes in *A. niger* and their expression levels in the Δ*lraA*, Δ*lraB*, Δ*lraC*, Δ*rhaR* mutants and the reference strain after a 2 hours transfer to L-rhamnose. **(See below)**

Table S6 | Genes of *A. niger* encoding pectinolytic enzymes and their expression in Δ*lraA*, Δ*lraB*, Δ*lraC*, Δ*rhaR* mutants and the reference strain after a 2 hours transfer to L-rhamnose. **Available upon request from the author.**

Table S4 | Identification of candidate LraD genes in *A. niger*. The data are organized according to the fold changes (highest to smallest).
In bold are indicated the three LraD candidates selected. The expression levels are mean values of duplicate samples. Genes with values higher than 120 are considered highly expressed and marked dark grey. Genes with values lower than 20 are considered low expressed and marked light grey.
The fold change is the difference in expression between the reference strain and the deletion mutants. The cut-off for differential expression is fold change >1.5 (cells marked dark grey if up-regulated) and fold change 0.7> (cells marked light grey if down-regulated) and p-value <0.05 (*).

	[a]**Micro-array**			**RNA-seq** (from this study)								
NRRL3 number	**Glc.2h**	**Rha.2h**	**Rha.2h/Glc.2h**	**Ref**	**Δ*lraA***	**Δ*lraB***	**Δ*lraC***	**Δ*rhaR***	**Ref/Δ*lraA***	**Ref/Δ*lraB***	**Ref/Δ*lraC***	**Ref/Δ*rhaR***
NRRL3_03899 (Δ*lraD1*)	51.54	2045.05	39.67*	123.38	23.51	19.26	120.89	68.64	5.24*	6.40*	1.02	1.80
NRRL3_05649 (Δ*lraD*2)	123.19	3050.94	24.76*	599.79	82.40	103.19	213.56	186.81	7.28	5.81	2.81	3.21
NRRL3_06731 (Δ*lraD*3)	134.62	524.08	3.89*	413.75	451.87	257.45	350.37	481.61	0.92	1.61	1.18	0.86
NRRL3_06310	65.04	84.45	1.30	12.27	12.37	14.83	13.98	22.81	0.99	0.83	0.88	0.53*
NRRL3_08779	17.76	16.64	0.94						#N/A	#N/A	#N/A	#N/A
NRRL3_09832	29.92	23.00	0.76*	0.00	0.38	0.39	0.00	0.00	0.00	0.00	NA	NA
NRRL3_01716				0.00	0.00	0.27	0.14	0.41	NA	0.00	0.00	0.00
NRRL3_04286				5.18	8.29	5.37	8.83	8.11	0.62	0.96	0.59	0.64

[a]Manuscript in preparation

Table S5 | Putative transporter genes in *A. niger* and their expression levels in the Δ*lraA*, Δ*lraB*, Δ*lraC*, Δ*rhaR* mutants and the reference strain after a 2 hours transfer to L-rhamnose.
The expression levels are mean values of duplicate samples. Genes with values higher than 120 are considered highly expressed and marked dark grey. Genes with values lower than 20 are considered low expressed and marked light grey. The fold change is the difference in expression between the reference strain and the deletion mutants. The cut-off for differential expression is fold change >1.5 (cells marked dark grey if up-regulated) and fold change 0.7> (cells marked light grey if down-regulated) and p-value <0.05 (*).

L-rhamnose transporters in *A. niger from* (Slothaak *et al.,* 2016)

Gene name	[a]Protein ID	*A. niger* NRRL3	Ref	Δ*lraA*	Δ*lraB*	Δ*lraC*	Δ*rhaR*	Ref/Δ*lraA*	Ref/Δ*lraB*	Ref/Δ*lraC*	Ref/Δ*rhaR*
RhtA	1119135	NRRL3_03278	809.38	153.66	241.54	2.85	4.12	5.26*	3.35*	283.99*	196.45*
	1089440	NRRL3_06137	20.51	12.99	7.54	7.90	71.03	1.57	2.72	2.59	0.28*
	1096151	NRRL3_09860	502.11	30.02	40.87	24.92	24.46	16.72*	12.28*	20.14*	20.52*
	1142034	NRRL3_01652	189.44	442.53	294.78	232.60	145.46	0.42*	0.64	0.81	1.30
	1143191	NRRL3_03147	971.20	1363.49	1884.59	1558.96	1052.06	0.71	0.52	0.62	0.92
	1147409	NRRL3_10300	120.48	173.87	203.04	256.15	394.54	0.69	0.59	0.47*	0.30*
	1156895	NRRL3_02828	836.48	120.38	160.15	5.02	4.66	6.94*	5.22*	166.62*	179.50*
	1180703	NRRL3_00235	17.87	35.17	20.67	27.18	121.63	0.50*	0.86	0.66	0.14*

Chapter 3

2-Keto-3-deoxy-L-galactonate mediates the induction of genes involved in D-galacturonic acid utilization in *Aspergillus niger*

Alazi E, Khosravi C, Homan TG, du Pré S, Arentshorst M, Di Falco M, Pham TTM, Peng M, Aguilar-Pontes MV, Visser J, Tsang A, de Vries RP and Ram AFJ. *FEBS Letters* 2017; 591 (10): 1408–1418

Abstract

In *Aspergillus niger*, the enzymes encoded by *gaaA*, *gaaB* and *gaaC* catabolize D-galacturonic acid (GA) consecutively into L-galactonate, 2-keto-3-deoxy-L-galactonate, pyruvate and L-glyceraldehyde, while GaaD converts L-glyceraldehyde to glycerol. Deletion of *gaaB* or *gaaC* results in severely impaired growth on GA and accumulation of L-galactonate and 2-keto-3-deoxy-L-galactonate, respectively. Expression levels of GA-responsive genes were specifically elevated in the Δ*gaaC* mutant on GA as compared to the reference strain and other GA catabolic pathway deletion mutants. This indicates that 2-keto-3-deoxy-L-galactonate is the inducer of genes required for GA utilization.

Introduction

Pectins are heterogeneous plant cell wall polysaccharides rich in D-galacturonic acid (GA). They represent a natural carbon source for many saprotrophic fungi including *Aspergillus niger* [1, 2]. The *A. niger* genome contains 58 genes encoding pectin-degrading enzymes [2, 3]. GA, the most abundant uronic acid in pectin, is transported by *A. niger* into the cell via the transporter GatA [4] and then catabolized into pyruvate and glycerol by consecutive action of four enzymes: GaaA, D-galacturonate reductase; GaaB, L-galactonate dehydratase; GaaC, 2-keto-3-deoxy-L-galactonate aldolase; and GaaD, L-glyceraldehyde reductase [5-8] (Fig. 1A). This four-step GA catabolic pathway is evolutionarily conserved in Pezizomycotina fungi [5], and has been studied in detail in *Botrytis cinerea* [9] and *Trichoderma reesei* [10-13]. In *B. cinerea,* the first enzymatic step is catalyzed by two functionally redundant enzymes, BcGar1 and the *A. niger* GaaA orthologue BcGar2 [9]. In *T. reesei*, GA is converted into L-galactonate by TrGar1 [10]. In addition, GaaA and GaaD (LarA) of *A. niger* have been shown to be involved in D-glucuronate and L-arabinose catabolism, respectively [14, 15].

Degradation of plant cell wall polysaccharides and subsequent transport and catabolism of released sugars are tightly controlled [16]. Genes required for pectin degradation, GA transport and GA catabolism are subject to carbon catabolite repression via CreA [17, 18]. They are specifically induced in the presence of GA [5, 17] and are regulated by the GaaR/GaaX activator-repressor module [19, 20]. The conserved Zn(II)2Cys6 transcription factor GaaR is required for growth on GA and for the activation of the GA-responsive genes in both *B. cinerea* and *A. niger* [19, 21].

The mechanism of activation of transcription factors can be diverse, and possibly requires so-called inducer molecules. These inducer molecules are often metabolites related to the substrate [22]. Only a few examples of activation of a transcription factor via an inducer have been elucidated in fungi. Probably the best studied example is the Zn(II)2Cys6 transcription factor Gal4p that regulates galactose utilization in *Saccharomyces cerevisiae*. Gal4p is repressed under non-inducing conditions because the transcriptional activation domain of Gal4p is bound to the co-repressor Gal80p. In the presence of galactose and ATP (inducing conditions), the sensor protein Gal3p binds to the Gal4p/Gal80p complex leading to dissociation of Gal4p and subsequent Gal4p-dependent transcription [23-27]. In the regulation of leucine biosynthesis, the Zn(II)2Cys6 transcription factor Leu3p interacts directly with a metabolic intermediate. The middle domain of the Leu3p protein masks the C-terminal activation domain by an intramolecular interaction in the absence of α-isopropylmalate (α-IPM), a metabolic intermediate of the leucine biosynthesis pathway. In the presence of α-IPM, which accumulates during leucine starvation, this self-masking is prevented, resulting in active Leu3p and activation of leucine biosynthesis genes [28-30]. The Gal4p and Leu3p transcription factors localize to the nucleus regardless of the presence or absence of inducer molecules [31, 32].

On the other hand, the transcriptional activator AmyR, involved in starch degradation in *A. nidulans* and *A. oryzae*, is translocated from the cytoplasm to the nucleus only in the presence of its inducer isomaltose [33-35].

In *A. niger*, GA or a derivative of GA was suggested to act as an inducer required for the activation of GA-responsive genes [17]. In *B. cinerea*, BcGaaR was shown to translocate from the cytoplasm to the nucleus in response to such an inducer [21]. Previous studies of *A. niger* and *B. cinerea* mutants disrupted in GA catabolic pathway did not unambiguously identify a specific inducer [6-9]. In this study, we constructed GA catabolic pathway deletion mutants (Δ*gaaA*, Δ*gaaB*, Δ*gaaC* and Δ*gaaD*) to gain insight into regulation of GA-responsive genes in *A. niger*. Comparative analysis of these mutants indicates that 2-keto-3-deoxy-L-galactonate acts as the physiological inducer of the GA-responsive genes.

Materials and Methods

Strains, media and growth conditions

All strains used in this study are listed in Table S1. MA249.1 was obtained by transformation of N593.20 (*cspA1, pyrG$^-$, kusA::amdS*) [19] with a 3.8 kb *Xba*I fragment containing the *A. niger pyrG* gene, resulting in the full restoration of the *pyrG* locus.

Media were prepared as described previously [36]. Radial growth phenotype analyses were performed with minimal medium (MM) (pH 5.8) containing 1.5% (w/v) agar (Scharlau, Barcelona, Spain) and various carbon sources: 50 mM glucose (VWR International, Amsterdam, The Netherlands), D-fructose (Sigma-Aldrich, Zwijndrecht, The Netherlands), GA (Chemodex, St Gallen, Switzerland), L-rhamnose (Fluka, Zwijndrecht, The Netherlands), L-arabinose (Sigma-Aldrich, Zwijndrecht, The Netherlands) or glycerol (Glycerol 87% BioChemica AppliChem, Darmstadt, Germany), or 1% (w/v) polygalacturonic acid (PGA) (Sigma, Zwijndrecht, The Netherlands), apple pectin (AP) (Sigma-Aldrich, Zwijndrecht, The Netherlands) or galactan (Acros Organics, Geel, Belgium). Filter sterilized D-fructose or GA solution was added after autoclaving MM with agar. Other carbon sources were autoclaved together with the medium. The plates were inoculated with 5 µL containing 10^4 freshly harvested spores and cultivated at 30 °C for 7 days. For microtiter plate growth phenotype analysis, wells in a 96-well, flat bottom plate (Sarstedt AG & Co., Nümbrecht, Germany) were filled with 180 µL MM (pH 5.8) containing 55 mM GA as the sole carbon source, and 20 µL freshly harvested spores (7.5×10^5 spores ml^{-1}). The plate was incubated with lids in EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) at 30 ^{o}C. Lid temperature was set to 32 ^{o}C to prevent condensation on the lid. Optical density at 600 nm was measured every hour. The average OD from the GA-containing control wells was subtracted from the OD of the test wells and negative values were corrected as zero.

For gene expression and metabolic analyses, 10^8 freshly harvested spores were inoculated and grown in 100 mL complete medium (CM) (pH 5.8) with 2% (w/v) D-fructose for 16 h, and mycelia were harvested by filtration through sterile myracloth. For northern blot and metabolic analyses, pre-grown mycelia were washed twice with MM with no carbon source (pH 4.5) and 1.5 g (wet weight) mycelia were transferred and incubated in 50 mL MM (pH 4.5) with 50 mM D-fructose or 50 mM GA for 2 h. For metabolic analysis, 1.5 g (wet weight) mycelia were transferred and incubated in 50 mL MM (pH 4.5) with 50 mM GA for 55 h. Additionally, 30 g (wet weight) mycelia of SDP20.6 (Δ*gaaC*) were transferred and incubated in 1 L MM (pH 4.5) with 50 mM GA for 55 h. For RNA-seq analysis, pre-grown mycelia were washed with MM with no carbon source (pH 6) and 2.5 g (wet weight) were transferred to 50 mL MM (pH 6) with 25 mM GA and grown for 2 h. All incubations were carried out in a rotary shaker at 30 °C and 250 rpm.

Construction of gene deletion strains

Protoplast-mediated transformation of *A. niger*, purification of the transformants and genomic DNA extraction were performed as described [36].

To construct the deletion cassettes, 5' and 3' flanks of the *gaaA*, *gaaB*, *gaaC* and *gaaD* genes were PCR-amplified using the primer pairs listed in Table S2 with N402 genomic DNA as template. For all cloning experiments *Escherichia coli* strain DH5*α* was used. To create SDP22.1 (Δ*gaaA*), SDP21.5 (Δ*gaaB*) and SDP20.6 (Δ*gaaC*), gene deletion cassettes were made using MultiSite Gateway Three-fragment Vector Construction Kit (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. *A. oryzae pyrG* gene flanked by AttB1 and AttB2 sites was amplified by PCR using the primer pair listed in Table S2 and plasmid pMA172 [37] as template. *gaaA*, *gaaB* and *gaaC* deletion cassettes containing 5' and 3' flanks of the target genes with *A. oryzae pyrG* gene in between were obtained by restriction digestion. To create EA1.1 (Δ*gaaD*), 5' flank of *gaaD* was ligated into pJET1.2/ blunt cloning vector (Thermo Fisher Scientific, Carlsbad, CA, USA) and amplified in *E. coli*. Following plasmid isolation, the 5' flank was excised using restriction enzymes *Kpn*I and *Xho*I, ligated into *Kpn*I-*Xho*I opened pBluescript II SK(+) (Agilent Technologies, La Jolla, CA, USA) and amplified in *E. coli*. *A. oryzae pyrG* gene was obtained from plasmid pMA172 [37] by restriction digestion with *Hind*III and *Xho*I. Isolated pBluescript II SK(+) plasmid containing the 5' flank was opened with restriction enzymes *Xho*I and *Not*I, and the *A. oryzae pyrG* gene as *Xho*I-*Not*I fragment and *Hind*III-*Not*I fragment of the *gaaD* 3' flank were ligated into the plasmid. Ligation product was amplified in *E. coli* and the linear deletion cassette was obtained by PCR amplification from the plasmid using primers gaaDP1-KpnI and gaaDP4-NotI. Deletion cassettes were introduced into the *pyrG*$^{-}$ strain N593.20. Gene deletions were confirmed via Southern blot analysis.

Gene expression analysis

Northern blot and RNA-seq analyses were performed as described [19] with minor modifications: For northern blot analysis, total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Probes were PCR-amplified using the N402 genomic DNA and the primer pairs listed in Table S2.

Chemical analysis

One mL culture samples were taken 7, 24, 31, 48 and 55 h after the transfer of mycelia to MM with GA. 250 µL of each culture sample was centrifuged at 16,000 *g* for 30 min and the supernatant was transferred to a new microfuge tube. After adding 1X volume of cold methanol (-20 °C), the sample was incubated on ice for 15 min and centrifuged at 16,000 *g* for 30 min. The supernatant was collected in a new microfuge tube and 1X volume of 0.1% formic acid was added. Metabolites in the extracellular culture fluids were analyzed by high pressure liquid chromatography - high resolution mass spectrometry. Aliquots were loaded, using a Series 200 micropump (Perkin Elmer, Waltham, MA), onto a reversed-phase Eclipse C18 2.1 x150 mm column (Agilent, Santa Clara, CA) connected in-line to a 7 Tesla LTQ-FT-ICR mass spectrometer (Thermo Electron Corporation, San Jose, CA) and negative mode electrospray ionization spectra were acquired at a resolution of 100000 at 200 m/z. Absolute GA concentration was calculated using a standard dilution calibration curve of commercially obtained GA (Chemodex, St Gallen, Switzerland). Standards for L-galactonate and 2-keto-3-deoxy-L-galactonate were not available, therefore these metabolites were assigned based on accurate mass alone (matched within a 5 ppm m/z window) and relative amounts in terms of extracted ion chromatograms peak areas were compared. One litre culture of SDP20.6 (Δ*gaaC*) was filtered through sterile myracloth 55 h after the transfer of mycelia to MM with GA, and the filtrate was stored at -80 °C. After freeze-drying, dry materials from SDP20.6 (Δ*gaaC*) extracellular culture fluid were dissolved in D_2O (Sigma Aldrich) for structural investigation by Nuclear Magnetic Resonance Spectroscopy (NMR). Spectra were recorded with a Varian VNMRS-500 MHz at 25 °C. The presence of 2-keto-3-deoxy-L-galactonate was confirmed by ^{1}H-NMR and ^{13}C-NMR.

Bioinformatics

RNA-seq data were analysed as described previously [19]. Differential expression was identified by Student's t-test with a *P*-value cut-off of 0.05. RNA-seq data for FP-1132.1 (reference strain) and SDP20.6 (Δ*gaaC*) were submitted to Gene Expression Omnibus [38] with accession numbers GSE80227 [19] and GSE95776 (this study), respectively.

Results

Growth analysis of D-galacturonic acid catabolic pathway deletion mutants

A. niger GA catabolic pathway deletion mutants, Δ*gaaA*, Δ*gaaB*, Δ*gaaC and* Δ*gaaD*, were constructed and were verified by Southern blot analysis (Fig. S1). We compared the growth phenotype of the strains on monomeric and polymeric carbon sources (Fig. 1, Fig. S2). Disruption of *gaaA* and *gaaD* resulted in reduced growth and sporulation on plates containing GA or PGA as carbon source. However, both mutants showed better growth on plates containing MM with GA compared to plates containing MM with no carbon source, indicating that they can still metabolize GA. The Δ*gaaB* and Δ*gaaC* mutants showed a more drastically reduced growth on plates containing GA, PGA or AP (Fig. 1B). The growth defects of the GA catabolic pathway deletion mutants on GA plates were confirmed in microtiter plate-based growth assays (Fig. 1C, Fig. S2A). None of the GA catabolic pathway deletion mutants exhibited defects in growth on other carbon sources tested, except that the deletion of *gaaD*, also known as the L-arabinose reductase gene *larA*, resulted in a poor growth on L-arabinose (Fig. S2B), confirming previous observations [15]. The inability of Δ*gaaB* or Δ*gaaC* to use GA as a carbon source suggests that there are no functionally redundant enzymes capable of replacing GaaB and GaaC.

Δ*gaaB* and Δ*gaaC* accumulate the D-galacturonic acid catabolic pathway intermediates L-galactonate and 2-keto-3-deoxy-L-galactonate, respectively

Since the roles of GaaB and GaaC in GA catabolism cannot be replaced by redundant enzymes, we expect the accumulation in the medium of the corresponding enzyme substrate in Δ*gaaB* and Δ*gaaC*, as shown previously [7, 8]. The extracellular GA concentration and the extracellular metabolites were examined by FT-ICR mass spectrometry over time during growth in GA. This analysis revealed that the reference strain utilized all GA in the medium within 48 h of growth, whereas in the GA catabolic pathway deletion mutants GA was still present in the medium after 55 h of growth (Fig. 2A).

In Δ*gaaA* and Δ*gaaD*, the concentration of GA gradually decreased to approximately 45% of the initial GA concentration in the medium, which reflects the slow catabolism of GA in these mutants. Δ*gaaB* consumed about 35% of the initial GA in 55 h and secreted L-galactonate. The time course consumption of GA by Δ*gaaB* was proportional to its release of L-galactonate (Fig. 2A). The Δ*gaaC* mutant took up about 78% of the initial GA in 55 h, and extracellular 2-keto-3-deoxy-L-galactonate accumulated in the medium of the Δ*gaaC* mutant over time (Fig. 2A). The presence of 2-keto-3-deoxy-L-galactonate in the extracellular culture fluid of the Δ*gaaC* mutant was confirmed by structural resolution by ^{1}H-NMR and ^{13}C-NMR (Fig. S3).

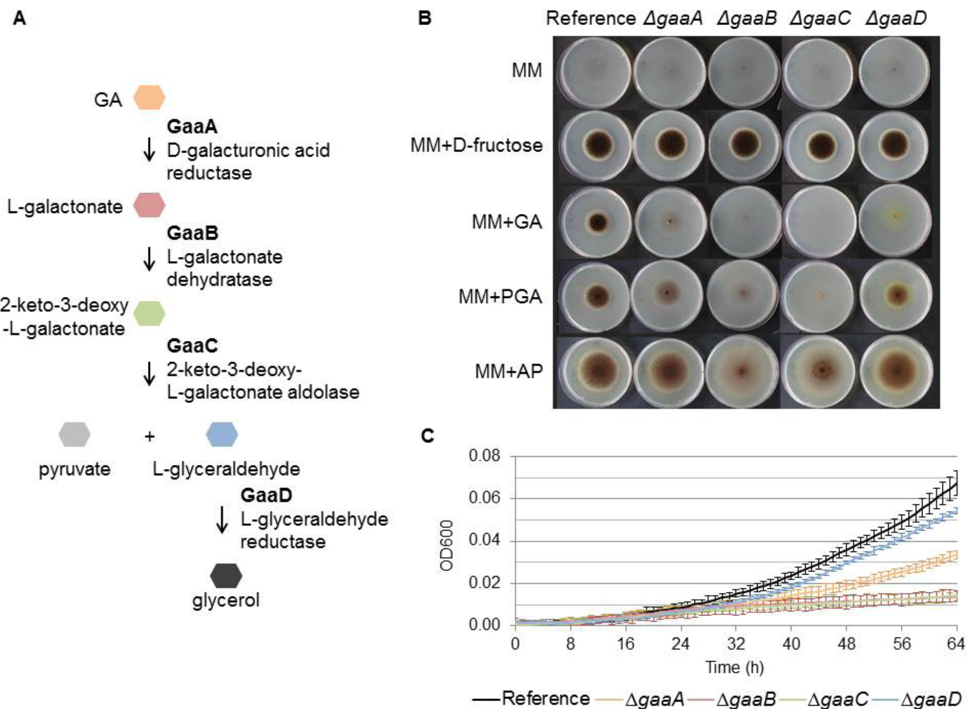

Figure 1 | (A) The evolutionarily conserved GA catabolic pathway in filamentous fungi as proposed by Martens-Uzunova and Schaap, 2008 [5]. GA is converted in pyruvate and glycerol by consecutive action of GaaA, GaaB, GaaC and GaaD enzymes. Growth profile of the reference strain (MA249.1) and GA catabolic pathway deletion mutants *ΔgaaA*, *ΔgaaB*, *ΔgaaC and ΔgaaD* (B) on solid MM without any carbon source, or with 50mM monomeric or 1% polymeric carbon sources after 7 days at 30 °C, and (C) in microtiter plate in liquid medium with 50 mM GA at 30 °C. Error bars represent standard deviation of six biological replicates.

Expression of D-galacturonic acid-responsive genes is increased in Δ*gaaC*

Genes involved in the degradation of the pectic substructures PGA (e.g., NRRL3_03144 exo-polygalacturonase and *pgx28B*) and rhamnogalacturonan I (RG-I) (e.g., NRRL3_10865 alpha-N-arabinofuranosidase), GA transport (*gatA*) and GA catabolism (*gaaA-D*) have been shown to be induced in the presence of GA [5, 18] and are part of the proposed GaaR/GaaX-controlled gene regulon [20]. To test the effect of the GA catabolic pathway gene deletions on the induction of GA-responsive genes, northern blot analysis was performed. The reference and Δ*gaaA*, Δ*gaaB*, Δ*gaaC* and Δ*gaaD* strains were pre-grown in D-fructose medium and transferred to either GA or D-fructose medium. Rapid induction of *gatA*, *gaaA*, *gaaB*, *gaaC*, *gaaD*, and NRRL3_10865 was observed in the reference strain upon transfer from D-fructose to GA as expected (Fig. 2B). Induction of these genes upon transfer to GA was also found in Δ*gaaA*, but at lower levels compared to the reference strain. The induction of GA-responsive genes was nearly absent in Δ*gaaB*. As shown in Fig. 2B, deletion of *gaaC* resulted in a hyper-induction of GA-responsive genes, especially pectinases (NRRL3_03144, *pgx28B* and NRRL3_10865).

Expression of *gatA*, *gaaA*, *gaaB*, *gaaC* and the pectinases in Δ*gaaD* was similar to the expression in the reference strain (Fig. 2B).

Transcriptome analysis of Δ*gaaC*

In order to analyze the expression of a larger number of genes controlled by GaaR/GaaX activator-repressor module in Δ*gaaC*, a genome-wide gene expression analysis was performed using RNA-seq. The reference strain and the Δ*gaaC* mutant were pre-grown in D-fructose medium and transferred to GA medium. Seventeen of the 53 GaaR/GaaX panregulon genes were significantly upregulated (FC≥2 and *P*-value≤0.05) in the Δ*gaaC* mutant cultured in GA as compared to the reference strain (Table 1, Table S3). These 17 genes include *gaaA* and six pectinases (NRRL3_03144, *pgx28B*, NRRL3_05252, NRRL3_04916, NRRL3_10559 and NRRL3_11738), as well as genes encoding four transporters and six genes for which the function has not yet been established. The expression of 24 of the remaining GaaR/GaaX panregulon genes was higher in Δ*gaaC* compared to the reference strain, but differences were relatively small and did not pass the stringent *P*-value of ≤0.05.

3

In addition to GaaR/GaaX-controlled genes, we also compared the expression of all 58 pectinases identified in the genome of *A. niger* [2] between the reference strain and the Δ*gaaC* mutant (Table S4, Fig. 2C). Apart from the six pectinases that depend on GaaR for induction [19], nine additional pectinases acting on the RG-I backbone, arabinan and arabinogalactan side chains were significantly upregulated (FC≥2 and *P*-value≤0.05) in the Δ*gaaC* mutant compared to the reference strain (Table 2). It has been reported that many of these genes are regulated by transcription factors RhaR (NRRL3_02832, NRRL3_07501, NRRL3_07501 and *faeB*), XlnR (NRRL3_05407 and *lac35B*) or AraR (*lac35B*), which are required for the utilization of L-rhamnose, xylan/D-xylose and arabinan/L-arabinose, respectively [39-42]. To address the possibility that deletion of *gaaC* affected the expression of these genes via their specific transcription factors, the expression of *rhaR*, *xlnR* and *araR* was analysed in more detail. Expression of *rhaR* (FC=5.84 and *P*-value=4.76E-03) and *xlnR* (FC=2.68 and *P*-value=5.60E-03) was significantly higher in Δ*gaaC*, which might explain the upregulation observed in these genes. The *araR* gene was not significantly differentially regulated in the Δ*gaaC* mutant.

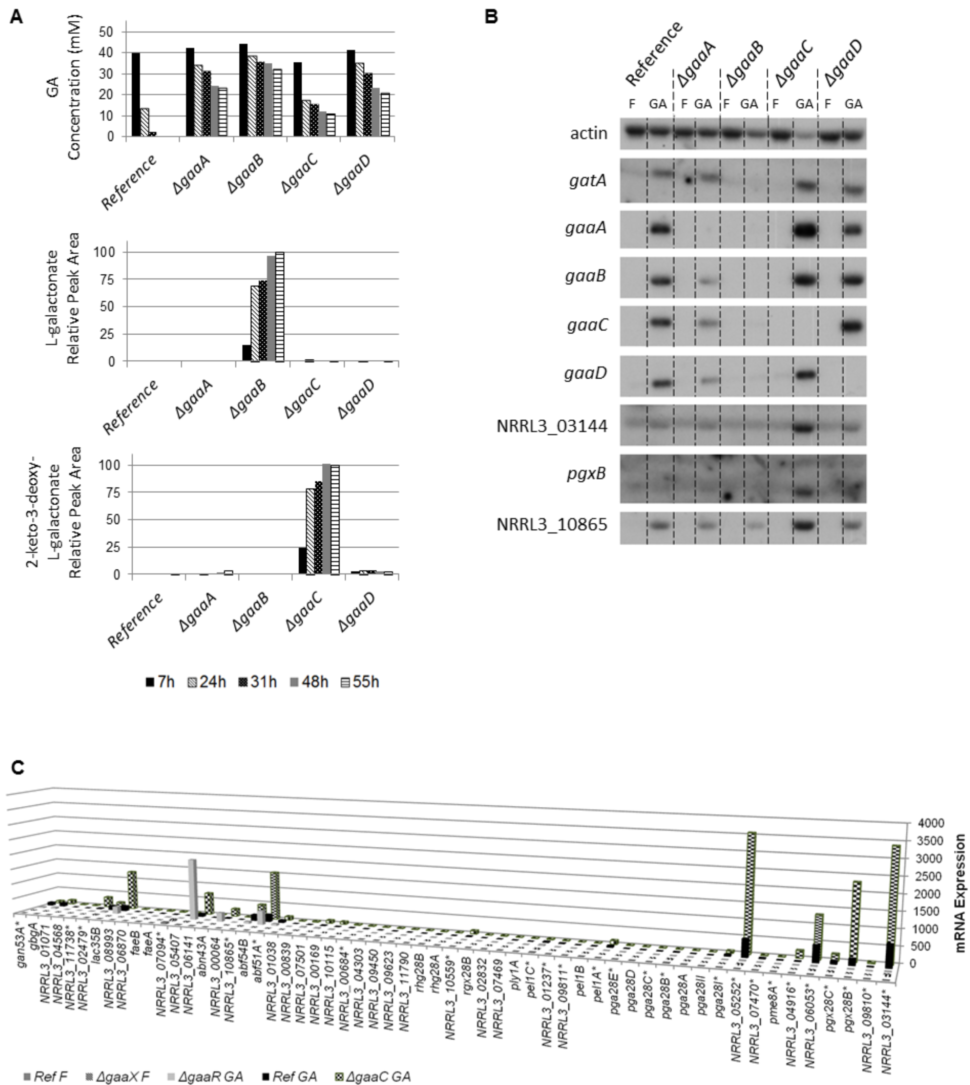

Figure 2 |Metabolic and gene expression analyses of *A. niger* GA catabolic pathway deletion mutants Δ*gaaA*, Δ*gaaB*, Δ*gaaC and* Δ*gaaD* (A) Extracellular GA, L-galactonate and 2-keto-3-deoxy-L-galactonate concentration in cultures of the reference strain (FP-1132.1) and GA catabolic pathway deletion mutants. GA concentration is given in mM and L-galactonate and 2-keto-3-deoxy-L-galactonate amounts are presented as ion chromatogram peak areas relative to Δ*gaaB* 55 h and *ΔgaaC* 55 h samples, respectively. (B) Northern blot analysis of selected GA-responsive genes in the reference strain (MA249.1) and GA catabolic pathway deletion mutants. Actin (NRRL3_03617) was used as a control. (C) RNA-seq analysis of pectinase genes in the reference strain (FP-1132.1) and Δ*gaaC* in GA (FPKM). Expression in Δ*gaaR* in GA (FPKM) [19], in the reference strain (MA234.1) and Δ*gaaX* in D-fructose (TPM) [20] was shown for comparison. Pectinase genes that belong to the GaaR/GaaX panregulon [20] are indicated with an asterisk. Strains were pre-grown in CM with 2% D-fructose. For metabolic analysis, mycelia were transferred to and grown in MM containing 50 mM GA. For northern blot analysis, mycelia were transferred to and grown in MM containing 50 mM D-fructose (F) or GA for 2 h. For RNA-seq analysis, mycelia were transferred to and grown in MM containing 25 mM GA for 2 h.

Discussion

In this study, we used GA catabolic pathway deletion mutants to investigate the induction mechanism of the GA-responsive genes in *A. niger*. We observed that the *gaaA* and the *gaaD* deletion mutants show reduced growth on GA or PGA compared to the reference strain, whereas growth of *ΔgaaB* and Δ*gaaC* is more severely reduced on GA, PGA or AP (Fig. 1B, Fig. 1C). These results are in line with the previous reports showing the inability of Δ*gaaB* and Δ*gaaC* to grow on GA [7, 8]. *ΔgaaA* was reported to be unable to grow on GA in a previous study [6], where the tenuous growth of Δ*gaaA* could have been interpreted as no growth. GA catabolic pathway deletion mutants derived from N593.20 in this study and from ATCC1015 in previous studies [6-8] showed the same growth defects on GA (unpublished results), excluding the possibility of a phenotypic difference caused by strain background. Deletion of *gaaB* and *gaaC* severely impaired growth on MM containing GA (Fig. 1B and C), indicating that there are no alternative enzymes replacing GaaB and GaaC. The residual growth of Δ*gaaA* and Δ*gaaD* on GA indicates that GA is catabolized in these reductase deletion mutants via partially redundant enzymes. In *B. cinerea*, there are two non-homologous D-galacturonate reductases, BcGar1 and BcGar2. While single gene deletion mutants (Δ*Bcgar1* or Δ*Bcgar2)* could still grow on GA, the double gene deletion mutant Δ*Bcgar1*Δ*Bcgar2* showed a complete loss of growth [9]. *A. niger* also contains a BcGar1 orthologue, NRRL3_06930, which shows no protein homology to GaaA. As in *B. cinerea*, NRRL3_06930 might enable the residual growth of Δ*gaaA* on GA. However, the expression of NRRL3_06930 is considerably lower than the expression of *gaaA* in GA, and unlike the expression of *gaaA*, does not depend on GaaR or GaaX [19, 20]. It is also possible that the two dehydrogenases belonging to the GaaR/GaaX panregulon, NRRL3_03342 and NRRL3_09863, partially replace GaaA or GaaD.

The recently proposed model related to the regulation of GA-responsive gene expression [20] postulates that under non-inducing conditions the repressor GaaX inhibits the transcriptional activity of GaaR. The repressing activity of GaaX is suggested to be lost in the presence of an inducer and subsequent activation of GaaR, resulting in the induction of GA-responsive genes in *A. niger* [20]. The results of metabolic and northern blot analyses indicate that accumulation of 2-keto-3-deoxy-L-galactonate in Δ*gaaC* is responsible for the induction of the GA-responsive genes. In other words, the pathway intermediate 2-keto-3-deoxy-L-galactonate, and not GA or L-galactonate, is the physiological inducer of the GA-responsive genes in *A. niger* (Fig. 2A, Fig. 2B). In the Δ*gaaA* mutant, we postulate that GA is converted into L-galactonate via partially redundant enzymes (see above) and the 2-keto-3-deoxy-L-galactonate produced is enough for the induction of GA-responsive genes. However, this induction is lower compared to the reference strain (Fig. 2B). This result is supported by a previous finding that *gaaB* and *gaaC* were expressed at lower levels in Δ*gaaA* compared to the reference strain [6].

Table 1 | RNA-seq analysis of 53 genes of the GaaR-GaaX panregulon [20] in Δ*gaaC* in GA. 27 genes belonging to GaaR-GaaX core regulon [20] are written in bold. Expression values (FPKM) are averages of duplicates. Significantly up-regulated genes (FC≥2 and *P*-value≤0.05) are highlighted.

Gene ID NRRL3	Gene ID CBS513.88	Description[a]	Gene Name	Reference	Δ*gaaC*	Δ*gaaC*/Ref	*P*-value
NRRL3_00958	**An14g04280**	**D-galacturonic acid transporter GatA**	***gatA***	888.35	1062.68	1.20	6.95E-02
NRRL3_03144	**An12g07500**	**exo-polygalacturonase**		698.90	3384.63	4.84	1.34E-02
NRRL3_05260	**An02g12450**	**exo-polygalacturonase Pgx28C**	***pgx28C***	99.93	192.85	1.93	9.11E-02
NRRL3_05649	**An02g07720**	**2-keto-3-deoxy-L-galactonate aldolase GaaC**	***gaaC***	5658.32	14.60	0.00	2.88E-04
NRRL3_05650	**An02g07710**	**D-galacturonic acid reductase GaaA**	***gaaA***	2599.98	6710.72	2.58	1.04E-02
NRRL3_06053	**An02g02540**	**carbohydrate esterase family 16 protein**		522.81	1301.08	2.49	8.01E-02
NRRL3_06890	**An16g05390**	**L-galactonate dehydratase GaaB**	***gaaB***	11309.00	13990.90	1.24	1.91E-01
NRRL3_08281	**An03g06740**	**exo-polygalacturonase Pgx28B**	***pgx28B***	200.31	2306.06	11.51	2.82E-02
NRRL3_08663	**An03g01620**	**MFS-type sugar/inositol transporter**		106.09	227.29	2.14	1.71E-01
NRRL3_10050	**An11g01120**	**L-glyceraldehyde reductase GaaD**	***gaaD***	8104.43	7499.78	0.93	5.79E-01
NRRL3_10865	**An08g01710**	**alpha-N-arabinofuranosidase**		201.62	440.98	2.19	1.92E-01
NRRL3_01237	**An19g00270**	**pectin lyase**		18.95	3.68	0.19	9.55E-03
NRRL3_02479	**An01g10350**	**exo-beta-1,4-galactanase**		137.63	170.01	1.24	5.21E-01
NRRL3_05252	**An02g12505**	**pectin methylesterase**		558.37	3569.08	6.39	2.07E-02
NRRL3_07470	**An04g09690**	**pectin methylesterase**		30.16	12.81	0.42	4.22E-02
NRRL3_08325	**An03g06310**	**pectin methylesterase Pme8A**	***pme8A***	6.54	6.74	1.03	8.79E-01
NRRL3_10559	**An18g04810**	**glycoside hydrolase family 28 protein**		20.00	97.18	4.86	1.19E-02
NRRL3_00965	An14g04370	pectin lyase Pel1A	*pel1A*	56.54	113.40	2.01	3.58E-01
NRRL3_04281	An07g00780	MFS-type transporter		90.41	106.00	1.17	5.05E-01
NRRL3_09810	An11g04040	exo-polygalacturonase		10.65	35.99	3.38	7.58E-02

NRRL3_08194	**An04g00790**	**Repressor of D-galacturonic acid utilization**	***gaaX***	381.34	529.21	1.39	1.97E-01
NRRL3_00684	An14g01130	rhamnogalacturonan lyase		5.77	13.23	2.29	2.61E-01
NRRL3_01606	An01g00330	alpha-N-arabinofuranosidase Abf51A	*abf51A*	87.96	111.63	1.27	4.97E-01
NRRL3_02571	An01g11520	endo-polygalacturonase Pga28I	*pga28I*	56.38	59.67	1.06	5.83E-01
NRRL3_02835	An01g14670	endo-polygalacturonase Pga28E	*pga28E*	4.26	13.51	3.17	9.99E-02
NRRL3_04153	An15g07160	pectin lyase		35.48	19.78	0.56	3.56E-02
NRRL3_04916	An07g08940	carbohydrate esterase family 16 protein		13.41	221.16	16.49	4.37E-02
NRRL3_05859	An02g04900	endo-polygalacturonase Pga28B	*pga28B*	15.10	4.12	0.27	9.36E-02
NRRL3_07094	An16g02730	endo-1,5-alpha-arabinanase		4.57	3.48	0.76	2.43E-01
NRRL3_08805	An05g02440	endo-polygalacturonase Pga28C	*pga28C*	5.26	7.27	1.38	1.85E-01
NRRL3_09811	An11g04030	pectin lyase		0.51	0.11	0.21	6.88E-02
NRRL3_10643	An18g05940	arabinogalactanase Gan53A	*gan53A*	105.64	67.21	0.64	2.70E-01
NRRL3_11738	An06g00290	beta-galactosidase		28.91	319.96	11.07	4.60E-02
NRRL3_00502	An09g06200	hypothetical protein		14.07	41.41	2.94	1.16E-01
NRRL3_00660	**An14g00860**	**carboxylesterase**		74.22	825.36	11.12	4.58E-02
NRRL3_00957	An14g04260	B3/B4 domain-containing protein		7.87	13.03	1.66	2.87E-01
NRRL3_01073	An14g05840	O-methyltransferase, COMT-type		3.22	11.45	3.55	1.39E-02
NRRL3_01127	**An14g06500**	**dihydroxyacetone kinase**		584.25	203.94	0.35	1.55E-02
NRRL3_01398	**An13g02090**	**MFS-type transporter**		26.10	96.31	3.69	1.69E-02
NRRL3_02770	An01g13880	MFS-type transporter		3.71	6.43	1.73	9.57E-02
NRRL3_03291	An12g05600	heterokaryon incompatibility protein		0.80	6.04	7.60	6.39E-02
NRRL3_03292	An12g05590	carboxylesterase		0.25	1.72	6.88	3.30E-01
NRRL3_03342	**An12g04990**	**short-chain dehydrogenase/reductase**		151.58	706.28	4.66	1.05E-02

NRRL3_03467	An12g03550	MFS-type transporter	4.91	92.55	18.85	2.61E-02
NRRL3_06244	**An02g00140**	**glycoside hydrolase family 43 protein**	80.90	137.44	1.70	1.81E-01
NRRL3_07382	An16g00540	alpha-L-fucosidase	2.29	8.06	3.53	4.41E-02
NRRL3_08499	An03g03960	uncharacterized protein	13.64	45.86	3.36	6.05E-03
NRRL3_08833	**n.a.**	**hypothetical protein**	4.29	1.87	0.44	2.27E-02
NRRL3_09862	An11g03510	hypothetical protein	0.43	0.20	0.45	5.62E-01
NRRL3_09863	**An11g03500**	**alpha-hydroxy acid dehydrogenase, FMN-dependent**	59.53	64.98	1.09	2.85E-01
NRRL3_10558	**An18g04800**	**alpha-L-rhamnosidase**	17.04	109.06	6.40	3.54E-02
NRRL3_11054	**An08g04040**	**MFS-type sugar/inositol transporter**	693.37	4713.62	6.80	8.89E-03
NRRL3_11710	An06g00620	MFS-type sugar/inositol transporter	341.35	1977.10	5.79	2.76E-02

Table 2 | RNA-seq analysis of 9 pectinase genes that were significantly upregulated in Δ*gaaC* in GA and do not belong to the GaaR-GaaX panregulon [20].

Gene ID NRRL3	Gene ID CBS513.88	Description[a]	Gene Name	Ref	Δ*gaaC*	Δ*gaaC*/Ref	*P*-value
NRRL3_02832	An01g14650	glycoside hydrolase family 28 protein		1.49	12.95	8.69	1.21E-02
NRRL3_09450	An11g08700	endo-rhamnogalacturonase		1.75	4.34	2.48	3.39E-02
NRRL3_07501	An04g09360	carbohydrate esterase family 12 protein		17.42	87.29	5.01	4.60E-02
NRRL3_00839	An14g02920	glycoside hydrolase family 105 protein		3.61	22.81	6.32	5.98E-03
NRRL3_05407	An02g10550	endo-1,5-alpha-arabinanase		103.20	702.79	6.81	1.45E-02
NRRL3_02931	An12g10390	feruloyl esterase FaeB	*faeB*	4.17	16.38	3.93	3.08E-02
NRRL3_02630	An01g12150	beta-galactosidase Lac35B	*Lac35B*	172.89	1259.38	7.28	3.28E-02
NRRL3_04568	An07g04420	exo-beta-1,4-galactanase		0.23	9.58	41.63	7.17E-03
NRRL3_01071	An14g05820	beta-galactosidase		0.75	8.06	10.74	2.90E-02

[a] Descriptions were obtained from manual annotation (manuscript in preparation).

In contrast, Δ*gaaB* possibly does not produce 2-keto-3-deoxy-L-galactonate from L-galactonate, since the growth phenotype of the Δ*gaaB* mutant suggests that there are no functionally redundant enzymes replacing GaaB. As a result, expression of GA-responsive genes is not induced in Δ*gaaB* (Fig. 2B). Reduced expression of *gatA*, *gaaA* and *gaaC* in the Δ*gaaB* mutant was also observed previously [7].
RNA-seq analysis of Δ*gaaC* revealed significant upregulation of several genes from the GaaR/GaaX panregulon involved in pectin breakdown and GA utilization, as well as genes with currently unknown link to GA utilization, such as transporters that might facilitate the faster GA transport in Δ*gaaC* compared to other GA catabolic pathway deletion mutants observed both in this study (Fig. 2A) and previous studies [6-8]. Deletion of *gaaC* also induced the expression of several pectinases acting on RG-I that do not belong to GaaR/GaaX panregulon (Table 2). A possible explanation is that starvation in Δ*gaaC* results in the induction of these genes. Several pectinases acting on side chains of RG-I, including NRRL3_05407, *lac35B* and NRRL3_07501, were previously reported to be induced upon starvation [43]. Another explanation is that the increased transcript levels of *rhaR* and *xlnR* results in an increase in the expression of these genes that were suggested to be under control of RhaR and XlnR.
Although both Δ*gaaB* and Δ*gaaC* cannot utilize GA, residual growth of Δ*gaaC* was observed on AP, whereas the growth of Δ*gaaB* on AP was more impaired (Fig. 1B). This could be explained by the high capacity of Δ*gaaC* to secrete pectinases acting on RG-I and release monosaccharides (L-arabinose, L-rhamnose, D-galactose) other than GA to support growth, and the less efficient pectinase production in Δ*gaaB*.

Previously, we identified 53 genes as the GaaR/GaaX panregulon downregulated in *ΔgaaR* under inducing condition and/or upregulated in Δ*gaaX* under non-inducing condition. However, only a core set of 27 genes was significantly differentially regulated under both conditions [19, 20], and only 17 of 53 panregulon genes, 10 of which belong to the core regulon, were hyper-induced in response to deletion of *gaaC* (Table 1), demonstrating the complex regulation of GA-responsive gene expression. A dynamic equilibrium is suggested to exist between the free and DNA-bound states of a transcription factor, and the binding of a transcription factor to the promoters of its target genes depends on its concentration, as well as its cooperative/competitive interactions with other proteins and the chromatin accessibility [44, 45]. Deletion of *gaaR* would result in the lack of GaaR in the cell, whereas deletion of *gaaX* or intracellular accumulation of 2-keto-3-deoxy-L-galactonate in Δ*gaaC* would, possibly to different degrees, increase the concentration of active GaaR by elimination or reducing the repressing activity of GaaX. GaaR concentration might also be regulated transcriptionally: *gaaX* is highly upregulated in GA [5], whereas *gaaR* expression is significantly increased in the Δ*gaaC* mutant (FC=5.10 and *P*-value=7.88E-03).

Moreover, different levels of CreA mediated repression on different GA-responsive genes [18] and accessibility of the promoter regions of these genes under different conditions might play a role in the observed differences in gene regulation. Condition specific cross-regulation between transcription factors and co-regulation of target genes might add additional complexity to GA-responsive gene expression, as discussed above.

To conclude, in this study we identified the GA catabolic pathway intermediate 2-keto-3-deoxy-L-galactonate as the probable inducer of the GA-responsive genes in *A. niger*. Considering that both the GA catabolic pathway enzymes and the GaaR/GaaX activator-repressor module is evolutionarily conserved in the Pezizomycotina subdivision of Ascomycetes [5, 20], it is highly probable that the mechanism by which 2-keto-3-deoxy-L-galactonate acts as an inducer and interacts with the activator-repressor module is also conserved.

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Chapter 4

Transcriptome analysis of *Aspergillus niger xlnR* and *xkiA* mutants grown on corn stover and soybean hulls

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Abstract

Enzymatic plant biomass degradation by fungi is a highly complex process and one of the leading challenges in developing a biobased economy. Some industrial fungi (e.g. *Aspergillus niger*) have a long history of use with respect to plant biomass degradation and for that reason have become 'model' species for this topic. *A. niger* is a major industrial enzyme producer that has a broad ability to degrade plant based polysaccharides. *A. niger* wild-type, the (hemi-)cellulolytic regulator (*xlnR*) and xylulokinase (*xkiA1*) mutant strains were grown on a monocot (corn stover) and dicot (soybean hulls) substrate. The *xkiA1* mutant is unable to utilize the pentoses D-xylose and L-arabinose and the polysaccharide xylan, and was previously shown to accumulate inducers for XlnR and AraR in the presence of pentoses, resulting in overexpression of their target genes. The *xlnR* mutant has reduced growth on xylan and down-regulation of its target genes. The mutants therefore have a similar phenotype on xylan, but an opposite transcriptional effect. D-xylose and L-arabinose are the most abundant monosaccharides after D-glucose in nearly all plant-derived biomass materials. In this study we evaluated the effect of the *xlnR* and *xkiA1* mutation during growth on two pentose-rich substrates, corn stover (CS) and soybean hulls (SBH), by transcriptome analysis.

Particular attention was given to CAZymes, metabolic pathways and transcription factors related to the plant biomass degradation. Genes coding for the main enzymes involved in plant biomass degradation were down-regulated at the beginning of the growth on CS and SBH. However, at a later time point, significant differences were found in the expression profiles of both mutants on CS compared to SBH.

This study demonstrates the high complexity of the plant biomass degradation process by fungi, by showing that mutant strains with fairly straightforward phenotypes on pure mono- and polysaccharides, have much less clear-cut phenotypes and transcriptomes on crude plant biomass.

Introduction

Aspergillus niger is a filamentous fungus that degrades plant biomass polysaccharides, such as cellulose, hemicellulose and pectin into monomeric sugars that can serve as a carbon source. Cellulose has a simple structure as a linear polymer of D-glucose. Hemicelluloses are more complex heterosaccharides with many variations in their structure. Pectins are a family of complex polysaccharides with D-galacturonic acid as the main monomeric component. The composition of plant biomass is detailed in Table 1. *A. niger* is able to secrete a broad spectrum of enzymes that can hydrolyze polysaccharides into pentoses, hexoses and other monomeric components [1], which can be taken up by the fungus. *A. niger* then uses a variety of catabolic pathways to efficiently convert the monomeric components of plant biomass. Significant progress has been made in the utilization and conversion of cellulose-derived hexose sugars into bioethanol. Several reports summarized the latest developments from 1st generation to 2nd generation (2G) ethanol technologies [2]. However, the use of pentose sugars, such as L-arabinose and D-xylose presents an opportunity to increase the efficiency of 2G bioethanol. In *A. niger* the release of L-arabinose and D-xylose from plant biomass requires the synergistic action of several Carbohydrate Active enZymes (CAZymes) [1]. After release from the polymers, L-arabinose and D-xylose are metabolized through the pentose catabolic pathway (PCP), consisting of oxidation, reduction and phosphorylation reactions to form D-xylulose-5-phosphate, which enters the pentose phosphate pathway (PPP) [3-5]. The PPP is one of the central metabolic pathways in primary carbon metabolism. The production of D-xylulose-5-phosphate from the PCP enables the fungus to answer efficiently to the increased demands of NADH and NADPH [6].

In *A. niger*, the xylanolytic enzyme system is regulated by the zinc binuclear transcription factor (TF) XlnR [5, 7-12]. In addition to extracellular enzymes, XlnR also regulates D-xylose reductase (*xyrA*) in the PCP, and ribose-5-isomerase (*rpiA*) and transaldolase (*talB*) in the PPP [13]. Activation of XlnR depends on the presence of D-xylose that acts as an inducer, released from the environment by low level constitutively expressed or starvation-influenced scouting enzymes [13-17]. It has been demonstrated that D-xylose induction is concentration-dependent: acting as an inducer for xylanases at low concentrations and as a repressor through CreA at higher concentrations [14, 18]. Another TF, AraR has been identified in *A. niger* and was shown to interact with XlnR in the regulation of the PCP [5, 13].

Corn stover (CS) and soybean hulls (SBH) are commonly used as renewable feedstocks for many applications. CS has strong advantages as a feedstock for energy, chemicals, and materials, because of its high volume and low cost [19]. CS contains stalks, leaves, tassel, husk, and cob from the corn crop [20], making it highly heterogeneous. The composition of each fraction varies, and each fraction is known to respond differently to enzymatic hydrolysis [21-23]. Crude CS consists of 37.1% cellulose, 20.9% hemicellulose, 13.5% lignin, and 1.3% ash [24].

Soybean hulls (SBH) is the predominant by-product from the soybean process industry [25]. The chemical composition of SBH may contain variable amounts of cellulose (29-51%), hemicellulose (10-25%), lignin (1-4%), pectin (4-8%), protein (11-15%), and minor extractives [25]. Lignin is the most recalcitrant component of the plant cell wall. SBH are easy degradable due to its low level of lignin and is therefore attractive as a potential feedstock for fuel and other industrial uses.

Different pretretment methods have been studied in relation to the production of monomeric sugars from CS and SBH [21, 26]. However, the costs of cellulase and hemicellulase production contribute significantly to the price of biofuel. Improving the methods to obtain these enzyme cocktails and increase their efficiency is a key factor to make biofuels economically sustainable. One of the possibilities to optimize the biofuel production process is the genetic engineering of enzyme production organisms, such as *A. niger.*

The role of XlnR in regulation of enzyme production was studied in detail on monosaccharides and polysaccharides, but the role of this TF on two natural substrates like CS and SBH has been studied less extensively. In this study we describe a transcriptomic analysis of *A. niger* wild-type, Δ*xlnR* and *xkiA1* mutant grown on CS and SBH. The goal was to analyze the effect of the deletion of *xlnR* and *xkiA1* over time during growth on these substrates. Our hypothesis in this study was that at an early time point the XlnR target genes would be down-regulated in Δ*xlnR* and up-regulated in *xkiA1* mutant due to accumulation of the inducers of XlnR and AraR. Previous studies demonstrated that transcript levels of several genes encoding cellulolytic, xylanolytic and xyloglucanolytic enzymes were decreased in an *xlnR* deletion mutant [10, 27, 28]. In contrast, increased transcript levels of genes encoding arabinan and xylan degrading enzymes have been observed in the *xkiA1* mutant, as well as intracellular accumulation of L-arabitol and xylitol [3, 5, 29]. At the later time points of our study , we expected *A. niger* to compensate for these mutations by using other regulatory mechanisms. Interestingly, our results demonstrated that the response of *A. niger* to crude plant biomass substrates is even more complex than could be extrapolated from studies on pure mono- and polysaccharides.

Materials and Methods

Strains, media and growth conditions

A. niger strains, CBS 141247 (N402, *cspA1*) [30], CBS 141248 (*cspA1,* Δ*argB, nicA1, leuA1,* Δ*xlnR*) [5] and CBS 141251 (N572, *cspA1, xkiA1, nicA1*) [31] were used in our study. All *A. niger* strains were grown at 30°C using minimal medium (MM, pH 6) or complete medium (CM, pH 6) [32] with 1.5% of agar. Spore plates contained CM with 2% D-glucose. Liquid cultures of three biological triplicates were inoculated with 10^6 spores/ml and incubated in a rotary shaker at 250 rpm and 30°C. Pre-cultures for RNA isolation were grown for 16h in 1L Erlenmeyer flasks containing 250 ml CM with 2% D-fructose. Mycelium was washed with MM and transferred for 4h, 24h and 48h, in 250 ml Erlenmeyer flasks containing 50 ml MM supplemented with 1% CS or 1% SBH for RNA-seq.

Table 1 |Composition of plant biomass. Based on Kowalczyk et *al.,* 2014 [53].

Biomass	Polymer	Monomers
Cellulose		D-glucose
Hemicellulose	Xylan	D-xylose
	Glucuronoxylan	D-glucuronic acid, D-xylose
	Arabinoglucuronoxylan	D-xylose, L-arabinose
	Arabinoxylan	D-xylose, L-arabinose
	Galacto(gluco)mannan	D-glucose, D-mannose, D-galactose
	Mannan/galactomannan	D-mannose, D-galactose
	Xyloglucan	D-glucose, D-xylose, D-fructose, D-galactose
	β(1,3)/(1,4)-Glucan	D-glucose
Pectin	Homogalacturonan	D-galacturonic acid
	Xylogalacturonan	D-galacturonic acid, D-xylose
	Rhamnogalacturonan I	D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose, ferulic acid, D-glucuronic acid
	Rhamnogalacturonan II	D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose, L-fucose, D-glucose, D-manno-octulosonic acid (KDO), D-lyxo-heptulosaric acid (DhA), D-xylose, D-apiose, L-acetic acid
Inulin		D-fructose, D-glucose
Starch	Amylose	D-glucose
	Amylopectin	D-glucose
Various gums		D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose, D-xylose, L-fucose (depending on the specific gum type)
Lignin		monolignols: ρ-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol

Mycelium was harvested after 4h, 24h and 48h by vacuum filtration, dried between tissue paper and frozen in liquid nitrogen.

RNA extraction, cDNA library preparation and RNA-sequencing

Total RNA was extracted from mycelium ground in a Tissue Lyser (QIAGEN) using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity and quantity were analyzed on a 1% agarose gel using gel electrophoresis and with the RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies). Stranded cDNA sequencing libraries were generated using the Illumina TruSeq Stranded mRNA HT sample prep kit utilizing poly-A selection of mRNA. The mRNA was purified from 1 ug of total RNA using magnetic beads containing poly-T oligos, and was then fragmented and reversed transcribed using random hexamers and SSII (Invitrogen) followed by second strand synthesis. The fragmented cDNA was treated with end-pair, A-tailing, Illumina index adapter ligation, and 8 cycles of PCR for template enrichment. The prepared libraries were 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 prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq Rapid paired-end cluster kit, v4. Sequencing of the flow cell was performed on the Illumina HiSeq2500 sequencer using HiSeq TruSeq SBS sequencing kits, v4, following a 1x100 indexed run recipe.

RNA data analysis

Raw fastq file reads were filtered and trimmed using the JGI QC pipeline. Using BBDuk (https://sourceforge.net/projects/bbmap/) raw reads were evaluated for artifact sequence by kmer matching (kmer=25), allowing 1 mismatch and detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method set at Q6. Reads under the length threshold were removed. Filtered reads from each library were aligned to the reference genome (http://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html) using HISAT version 0.1.4-beta [33], featureCounts [34] was used to generate the raw gene counts using gff3 annotations. On average 98% of the reads were mapped to the genome and 80% of the reads were mapped to the gene. Gene expression was calculated as FPKM (Fragments Per Kilobase of transcript per Million mapped reads). DESeq2 (version 1.10.0) [35] was used to determine which genes were differentially expressed between pairs of conditions. The parameters used to call a gene differently expressed between conditions were adjusted p-value < 0.05 and log2 fold change 0.6 for up-regulated and -0.6 for down-regulated. Raw gene counts were used for DGE analysis. DESeq2 uses its own internal normalization. The RNA-seq data have been submitted to Gene Expression Omnibus (GEO) [36] with accession number: (will be added later at the proof stage).

Results and discussion

Overall effect of *xlnR* deletion and *xkiA1* mutation on the CAZy genes involved in the plant biomass degradation

To gain more insight into the regulation of cellulose-, hemicellulose- and pectin-degrading enzymes by XlnR on a natural substrate, the wild-type strain and the mutant strains Δ*xlnR* and *xkiA1* were pre-grown in liquid cultures containing MM with D-fructose, and then transferred to MM with 1% CS or 1% SBH for 4, 24 and 48h. RNA-seq analysis was performed and the transcriptome response during growth on CS and SBH was analyzed in the mutants compared to the wild-type strain. Based on previous studies on monosaccharides and polysaccharides, it was expected that XlnR-target genes will be down-regulated in the *xlnR* mutant and up-regulated in the *xkiA1* mutant at the early time point [29]. The expression data were analyzed to evaluate whether this is also the case on a crude substrate consisting of multiple monomeric compounds. *A. niger* XlnR is involved in degradation of cellulose, xylan, xyloglucan and to some extent galactomannan [9-11, 37]. The *xkiA1* mutant is an UV mutant, unable to grow on L-arabinose and D-xylose and deficient in D-xylulose kinase activity [3, 29]. XkiA is essential for the utilization of D-xylose and L-arabinose, which are major components of xylan, xyloglucan and pectin. Since CS contains mainly cellulose and xylan, and SBH mainly cellulose, xyloglucan and pectin, we evaluated the effects of the deletion of *xlnR* and *xkiA1* on CAZy genes related to these polysaccharides.

Genes were considered differentially expressed if the log2 fold change was greater than 0.6 or less than -0.6 with p-value ≤0.05.The difference in CAZy gene expression of Δ*xlnR* and the *xkiA1* mutant compared to the wild-type was analyzed over time (4, 24 and 48h). After 4h on CS 108 genes were down-regulated in Δ*xlnR* and from those genes, two were up-regulated and 79 were down-regulated in the *xkiA1* mutant (Fig. 1, Table S1). Similar results were observed after 24h on CS, with 108 genes that were down-regulated in Δ*xlnR* of which four were up-regulated and 63 were down-regulated in the *xkiA1* mutant. After 48h on CS 108 genes were down-regulated in Δ*xlnR* and from them 23 were up-regulated and 47 were down-regulated in the *xkiA1* mutant, indicating that the highest number of CAZy genes showed the expected profile of down-regulated in the *xlnR* mutant and up-regulated in the *xkiA1* mutant at the latest time point.

Expression of a previously identified set of 21 XlnR-dependent targets genes was evaluated in our data-set (Table 2), most of which were significantly down-regulated in Δ*xlnR*. The exception was an α-rhamnosidase encoding gene (NRRL3_07520) after 4h of transfer to CS. Interestingly, after 24h of transfer to CS, of the four genes down-regulated in Δ*xlnR* and up-regulated in the *xkiA1* mutant, only one gene has been identified as an XlnR-target gene: β-xylosidase (BXL; *xlnD*) (Table 2). After 48h of transfer to CS, of the 23 genes that were down-regulated in Δ*xlnR* and up-regulated in the *xkiA1* mutant, two genes have been previously identified as XlnR-target genes: an α-galactosidase (AGL; *aglB*) and an α-xylosidase (AXL; *axlA*). Overall, the set of genes responding to the mutations differs from those observed on xylan or D-xylose, indicating the more complex regulatory system that is active during growth on crude plant biomass.

After 4h on SBH, 96 genes were down-regulated in Δ*xlnR* and of those genes six were up-regulated and 68 were down-regulated in the *xkiA1* mutant (Fig.1; Table S1). Compared to CS, there was a larger shift in the expression profiles between the time points, since after 24h on SBH, only 48 genes were down-regulated in the Δ*xlnR* strain of which eight were up-regulated and 12 were down-regulated in the *xkiA1* mutant. After 48h on SBH 67 genes were down-regulated in Δ*xlnR*. From these, 18 were up-regulated and six were down-regulated in the *xkiA1* mutant. As was observed for CS, after 48h the highest number of CAZy genes showed the expected profile of being down-regulated in the *xlnR* deletion mutant and up-regulated in the *xkiA1* mutant. One α-galactosidase (AGL; *aglB*), two cellobiohydrolases (CBH; *cbhA* and *cbhB*) and one endoglucanase (EGL; *eglA*) were down-regulated in Δ*xlnR* and up-regulated in the *xkiA1* mutant after 24h and 48h of transfer to SBH. In addition, *axlA* was down-regulated in Δ*xlnR* and up-regulated in the *xkiA1* mutant after 48h of transfer to SBH (Fig.1; Table S1).

Overall, larger differences were observed in SBH compared to CS after 24h and 48h. A higher number of CAZy genes were up-regulated in the *xkiA1* mutant, especially pectinases, on SBH compared to CS after 24h. Our results showed an antagonistic effect between Δ*xlnR* and the *xkiA1* mutant after 48h to CS and SBH, since more genes were up-regulated in the *xkiA1* mutant compared to Δ*xlnR*, while more genes were down-regulated in Δ*xlnR* compared to the *xkiA1* mutant.

Deletion of *xlnR* affects CAZy gene expression on CS and SBH

We first analyzed in detail the RNA-seq data generated from the strains grown on CS due to its high amount of cellulose and arabinoxylan and the major role of XlnR when *A. niger* grows on this substrate. Secondly, we looked at the differences or similarities between CS and SBH in both deletion mutants compared to the wild-type. Only genes differently expressed between conditions with p-value < 0.05 and a log2 fold change of at least 0.6 for up-regulated and -0.6 for down-regulated were considered in this analysis.

Expression of cellulolytic genes

After 4h and 24h of transfer to CS, 15 cellulolytic CAZy genes were down-regulated in Δ*xlnR* compared to the wild-type, while after 48h, 13 cellulolytic CAZy genes were down-regulated (Fig. 2-3; Table S1 and Fig. S1). Some cellulolytic genes were up-regulated in the Δ*xlnR* strain at all three tested time-points. In the *xkiA1* mutant after 4 and 24h a similar trend can be observed; most cellulolytic genes were down-regulated and only a few genes were up-regulated, but after 48h the opposite effect was observed. Two cellulolytic genes were down-regulated and ten were up-regulated in the *xkiA1* mutant compared to the wild-type. In SBH, the same trend as for CS was observed in Δ*xlnR*, in that the majority of cellulolytic genes were down-regulated at all the time points tested (Fig. 2-3; Table S1 and Fig. S1), but a lower number of genes were differentially expressed in the *xkiA1* mutant compared to CS. Several cellulolytic genes, previously identified as XlnR-target genes showed interesting transcript profiles. Two endoglucanases (EGL; *eglA* and *eglC*) [10, 37] were down-regulated at all time points in both substrates, while a third EGL, *eglB*, was only down-regulated after 24h in CS and after 4h in SBH. Two XlnR-regulated cellobiohydrolases (CBH; *cbhA* and *cbhB*) [11] were down-regulated at all the time points in CS, while in SBH, *cbhA* was down-regulated only after 4h and *cbhB* after 4h and 48h. Interestingly, *eglA*, *cbhA* and *cbhB* showed the expected profile, down-regulated in Δ*xlnR* and up-regulated in the *xkiA1* mutant, but only after 48h of transfer to CS and not at the earlier time points.

Expression of xylan and xyloglucan genes

At all time points tested in CS and SBH, the majority of the xylanolytic genes and xyloglucan-specific genes were down-regulated in Δ*xlnR*. After 4h in CS most of the xylanolytic genes and xyloglucan-specific genes were also down-regulated in the *xkiA1* mutant, but after 24h, the effect of the *xkiA1* mutation is less pronounced, and after 48h more xyloglucan- specific genes were up-regulated, compared to the earlier time points (Fig. 2-3; Table S1 and Fig. S1). No major differences were observed after 4h in SBH in the *xkiA1* mutant compared to Δ*xlnR*. After 24h, unlike in CS, no xylanolytic genes and xyloglucan-specific genes were down-regulated in SBH in the *xkiA1* mutant. After 48h no xylanolytic genes were down-regulated in SBH in the *xkiA1* mutant compared to the wild-type, whereas four were down-regulated in CS.

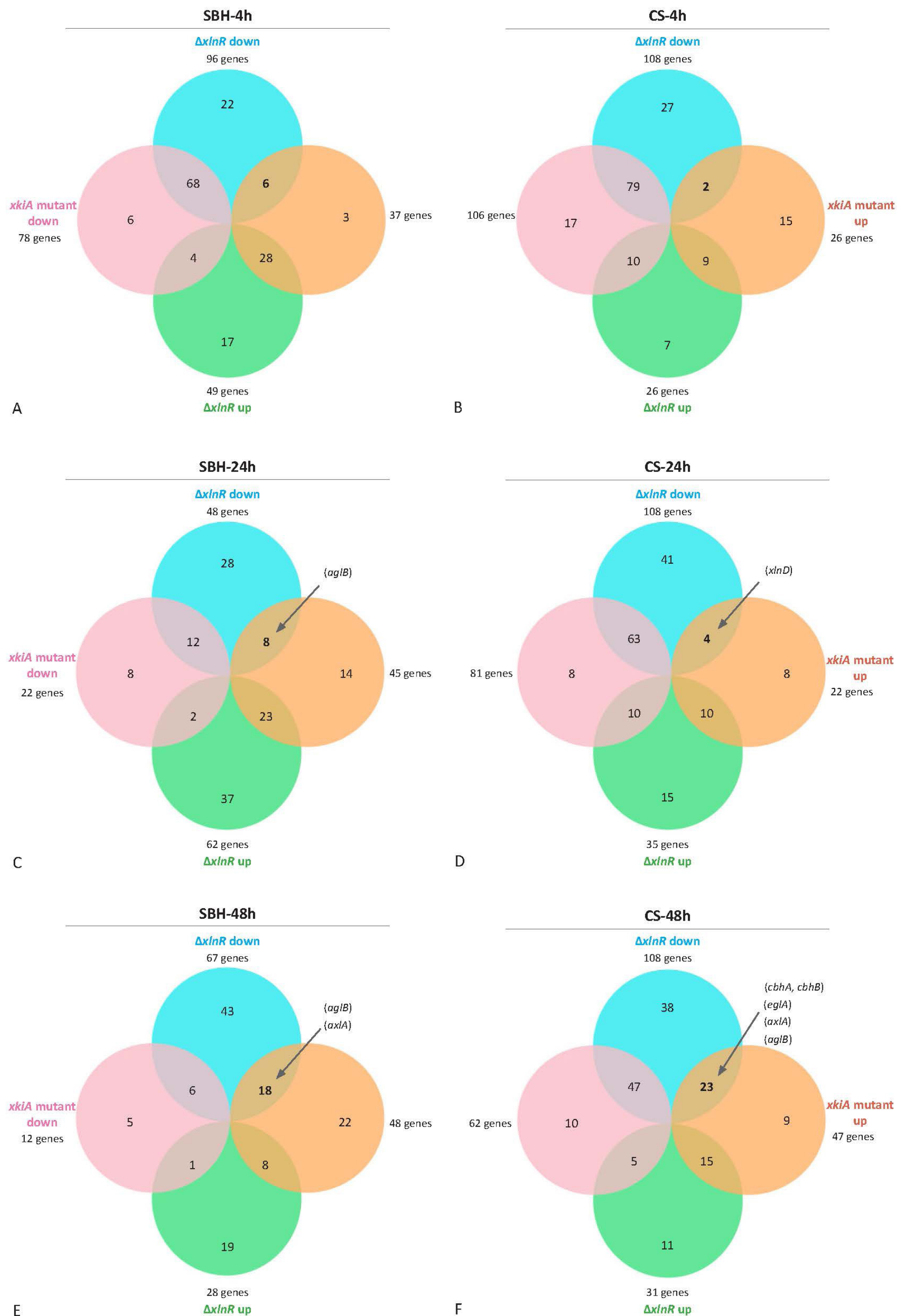

Figure 1 | Venn diagrams showing the CAZy genes involved in the degradation of plant biomass in *A. niger* that are significantly up-regulated and down- regulated genes in SBH (A, C, E) and CS (B, D, F) between Δ*xlnR* vs the wild-type (green and blue) and between *xkiA1* vs the wild-type (orange and pink) after 4h (A; B), 24h (C; D) and 48h (E,F). The gene numbers are listed in Table S1.

Table 2 | Genes regulated by XlnR in the *A. niger* Δ*xlnR* mutant compared to the wild-type after 4h, 24h, 48h of transfer to 1% corn stover (CS) or 1% soybean hulls (SBH). Down-regulated genes in the Δ*xlnR* mutant with a fold change <-0.6 and p<0.05 are indicated with (Down) and up-regulated genes with a fold change >0.6 and p<0.05 with (Up). Genes which are not differentially expressed are indicated with (No).

Enzyme code	Gene name	CAZy family	Gene number	4h CS	24h CS	48h CS	4h SBH	24h SBH	48h SBH	Reference
AXE	*axeA*	CE1	NRRL3_03339	Down	Down	Down	Down	Down	Down	[9, 10, 38]
FAE	*faeA*	CE1	NRRL3_00007	Down	Down	Down	Down	Down	Down	[9, 10, 38]
BGL		GH1	NRRL3_09976	Down	Down	Down	Down	Down	Down	[38]
BGL	*bglA*	GH3	NRRL3_10449	Down	Down	No	Down	Down	Down	[38]
XLN	*xlnC*	GH10	NRRL3_08708	Down	Down	Down	Down	Down	Down	[9, 10, 38]
XLN	*xlnB*	GH11	NRRL3_01648	Down	Down	Down	Down	Down	Down	[9, 10, 38]
MND	*mndB*	GH2	NRRL3_09051	Down	Down	Down	No	No	No	[38]
AGL	*aglB*	GH27	NRRL3_05358	Down	Down	Down	No	Down	Down	[8, 38]
BXL	*xlnD*	GH3	NRRL3_02451	Down	Down	Down	Down	Down	No	[9, 10, 38]
AXL	*axlA*	GH31	NRRL3_00268	Down	Down	Down	Down	Down	Down	[38]
LAC	*lacA*	GH35	NRRL3_02630	Down	Down	Down	Down	Down	Down	[8, 38]
BXL		GH43	NRRL3_10881	Down	Down	Down	Down	Down	Down	[38]
CBH		GH6	NRRL3_10870	Down	Down	Down	Down	Down	Down	[38]
AXH	*axhA*	GH62	NRRL3_08707	Down	Down	Down	Down	Down	Down	[9, 10, 38]
AGU	*aguA*	GH67	NRRL3_01069	Down	Down	Down	Down	Down	Down	[9, 10, 38]
RHA		GH78	NRRL3_07520	No	Down	Down	No	No	No	[38]
EGL	*eglA*	GH5	NRRL3_02585	Down	Down	Down	Down	Down	Down	[10]
EGL	*eglB*	GH5	NRRL3_06791	No	Down	Up	Down	No	No	[10]
EGL	*eglC*	GH5	NRRL3_04917	Down	Down	Down	Down	Down	Down	[37]
CBH	*cbhA*	GH7	NRRL3_04953	Down	Down	Down	Down	Up	No	[11]
CBH	*cbhB*	GH7	NRRL3_02584	Down	Down	Down	Down	Up	Down	[11]

Previously, two endoxylanases (XLN; *xlnA*, *xlnB*) and a β-xylosidase (BXL, *xlnD*) have been identified as XlnR-target genes [9, 10]. In our RNA-seq analysis, *xlnA* and *xlnB* were down-regulated at all time points in both substrates, while *xlnD* was also down-regulated at all time point in CS, but only after 4h and 24h in SBH. These genes were in general not up-regulated in the *xkiA1* mutant, with the exception that *xlnD* was up-regulated only after 24h on CS.

Expression of pectinolytic genes

At all the time points tested, most of the pectinolytic genes were down-regulated in CS in both Δ*xlnR* and the *xkiA1* mutant (Fig. 2-3; Table S1 and Fig. S1). In contrast, after 4h in SBH, ten pectinolytic genes were up-regulated, while only one was up-regulated in CS in Δ*xlnR*. This became even more pronounced after 24h, when twenty-nine pectinolytic genes were up-regulated in SBH, whereas only six were up-regulated in CS in Δ*xlnR*. In contrast, only four were down-regulated at this time point in SBH. Interestingly, this pattern changed after 48h, as then thirteen pectinolytic genes were down-regulated in SBH, and twenty-six were down-regulated in CS in Δ*xlnR*, and the number of up-regulated genes reduced to ten for SBH and seven for CS.

The pectinolytic expression profiles of the *xkiA1* mutant in CS and SBH after 24h were similar to Δ*xlnR,* with sixteen pectinolytic genes that were up-regulated in SBH, while only five were up-regulated in CS. However, unlike for Δ*xlnR,* this effect was still observed after 48h.

Overall, pectinolytic gene expression seems to go up in the absence of XlnR and to a smaller extent XkiA on SBH, which could be explained by the use of L-rhamnose and/or D-galacturonic acid as an alternative carbon source, which is highly present in this substrate. This would be expected to result in increased induction of GaaR and RhaR, two of the main activators of pectinolytic genes. Alternatively, these regulators may be under post-transcriptional control in the presence of these compounds, as was shown for XlnR on D-xylose [38].

Expression of CAZy genes related to other plant biomass components

The expression of CAZy genes related to other plant biomass components (galactomannan, starch, and inulin) was also evaluated to determine whether expression of these genes was affected in the mutants. At all time points in CS most of the galactomannan-specific genes, starch-specific genes and CAZy genes acting on various substrates were down-regulated in Δ*xlnR* (Fig. 2-3; Table S1 and Fig. S1). One galactomannan-specific gene, previously identified as XlnR-target genes, *aglB* [8], was up-regulated in SBH and down-regulated in CS. However, after 4h, four inulin-specific genes were up-regulated, while one was down-regulated in CS in Δ*xlnR.* Most of the galactomannan-specific genes and starch-specific genes were down-regulated in the *xkiA1* mutant in CS at all time points, but this was only the case at 4 and 24h in CS for the CAZy genes acting on various substrates.

After 4h in SBH, more starch-specific genes were up-regulated in Δ*xlnR* compared to CS. After 24h three galactomannan-specific genes and four inulin-specific genes were up-regulated in SBH, whereas only one of each group was up-regulated in CS. No major differences were observed after 48h between CS and SBH in Δ*xlnR.* In the *xkiA1* mutant, after 4h in SBH, more galactomannan-specific genes, inulin-specific genes and CAZy genes acting on various substrates were up-regulated compared to CS. This effect became less pronounced after 24 and 48h.

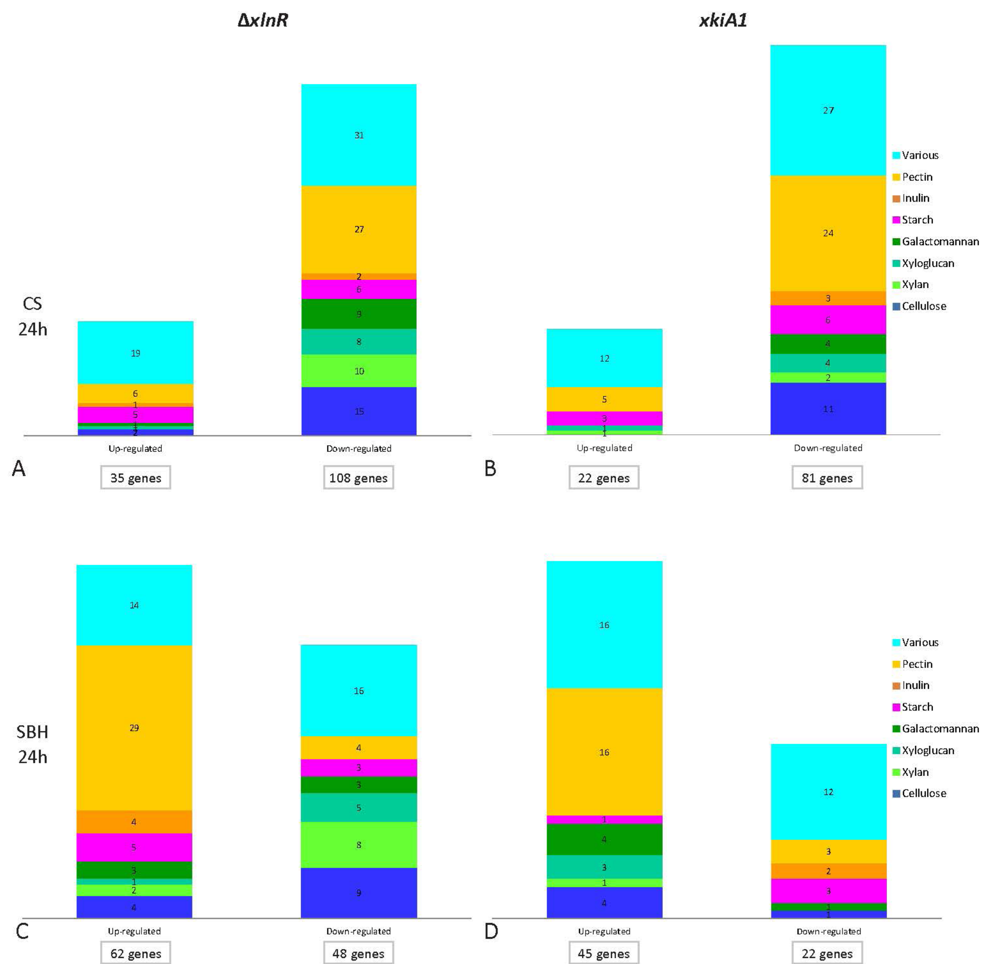

Figure 2 | CAZy genes involved in the degradation of plant biomass in *A. niger* that are significantly up-regulated or down-regulated between Δ*xlnR* vs the wild-type (A;C) and between *xkiA1* vs the wild-type (B;D) after 24h of transfer to CS (A;B) and SBH (C;D). The gene numbers are listed in Table S1.

These results showed that the effect of *xlnR* deletion and *xkiA1* mutation on CAZy gene expression changes over time and depends on the composition of the crude substrates. Overall, many CAZy genes involved in the degradation of cellulose, xylan and xyloglucan were down-regulated at all time points tested on both substrates in Δ*xlnR* (Fig. 2-3; Table S1 and Fig. S1). In the *xkiA1* mutant most of the cellulolytic, xylanolytic and xyloglucan-specific genes were down-regulated after 4h in both substrates as observed for Δ*xlnR*. After 4h in CS or SBH, *xlnR* and *xkiA1* mutants respond in a similar way, suggesting that at this early time point inability to use pentoses is the main effect on the expression profiles rather the difference in the mutation causing this. However, after 24h and 48h differences were observed in both deletion mutants between the two crude substrates. In the *xkiA1* mutant, a higher number of cellulolytic genes were down-regulated after 24h and up-regulated after 48h in CS, compared to SBH. Also, after 24h, more xylanolytic and xyloglucan-specific genes were down-regulated in SBH compared to CS in the *xkiA1* mutant. After 24h in SBH a high number of enzymes acting on the different substructures of the pectin, homogalacturonic acid (HGA), rhamnogalacturonan I (RG-I) and side chains (SC) were up-regulated in both mutants compared to CS.

After 48h, a high number of pectinases were up-regulated in SBH in the *xkiA1* mutant. Our data showed that the mutation of *xkiA1* results in up-regulation, whereas the deletion of *xlnR* results in down-regulation of several CAZymes involved in plant biomass degradation. This demonstrates that a metabolic and regulatory mutation with the same phenotype when grown on pure monosaccharides can result in a different physiology during prolonged growth on crude substrates.

Previously, we demonstrated the dominant role of XlnR in colonization and degradation of wheat bran [39]. During the late colonization stage (40h post inoculation), only the strains in which *xlnR* was deleted were unable to colonize the smooth surface of wheat bran, due to the absence/reduction of several cellulolytic and arabinoxylanolytic enzymes. These results correlate with the down-regulation of CAZymes involved in the degradation of cellulose, xylan, xyloglucan and galactomannan observed in the Δ*xlnR* strain on CS and SBH.

Expression profiles of other regulators involved in the degradation of CS and SBH and their metabolic target genes

The monomeric composition of CS and SBH is detailed in Table 3. CS and SBH contain various polysaccharides and provide options for consumption of other sugars than hexoses, for example pentoses (D-xylose and L-arabinose) and uronic acids. It is important to notice that the uronic acid level is higher in SBH than in CS and it also consists of different quantities of the other monomeric sugars. In SBH, the uronic acid fraction consists mainly of D-galacturonic-acid, while CS contains (4-(O)-methyl-)D-glucuronic-acid [1]. These differences in composition not only imply variation in the presence or levels of inducers for plant biomass related transcriptional regulators, but also the need –to activate different metabolic pathways in time to optimally use the two substrates.

To analyze the effect of *xlnR* or *xkiA1* mutants on sugar catabolism, expression of genes involved in conversion of L-arabinose/D-xylose, L-rhamnose, and D-galacturonic acid, and the regulators controlling them, was analyzed in the *xlnR* and *xkiA1* mutants compared to the wild-type strain grown on CS and SBH for 4h, 24h, and 48h. Expression of other TFs involved in the cellulose, hemicellulose and pectin degradation was also analyzed to determine the effect of *xlnR* or *xkiA1* mutants on their expression.

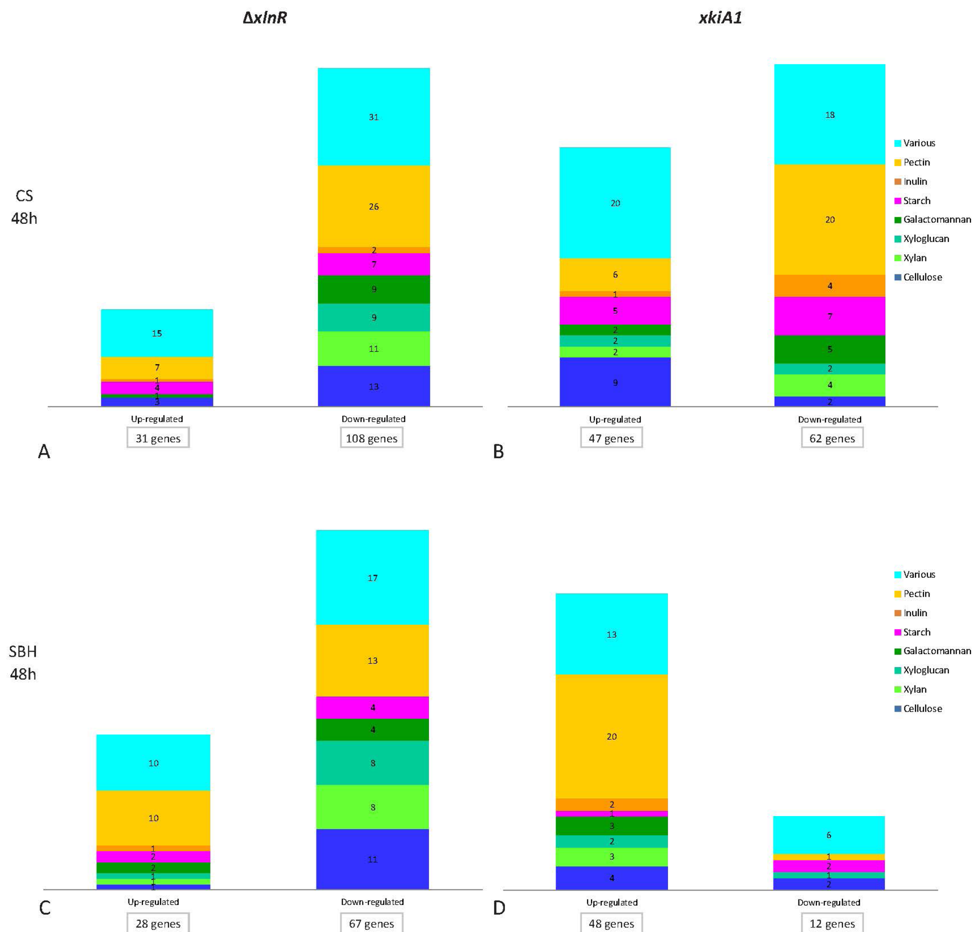

Figure 3 | CAZy genes involved in the degradation of plant biomass in *A. niger* that are significantly up-regulated or down-regulated between Δ*xlnR* vs the wild-type (A;C) and between *xkiA1* vs the wild-type (B;D) after 48h of transfer to CS (A;B) and SBH (C;D). The gene numbers are listed in Table S1.

Table 3 | Composition of the substrates used in this study.

Mol %	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Total
SBH	1.0	0.0	8.4	15.0	7.1	4.0	50.0	15.9	68.0
CS	0.4	0.0	4.6	34.9	0.7	1.7	53.4	4.3	59.5

The L-arabinose-responsive regulator AraR

AraR regulates most genes involved in the PCP: L-arabinose reductase (*larA*), L-arabitol dehydrogenase (*ladA*), L-xylulose reductase (*lxrA*), xylitol dehydrogenase (*xdhA*) and D-xylulokinase (*xkiA1*) during growth on L-arabinose in *A. niger* [5, 13]. The later two genes as well as D-xylose reductase (*xyrA*) are under control of XlnR during growth on D-xylose. In addition, *rpiA* and *talB* have been identified as XlnR regulated genes. After 4h of transfer to CS or SBH all the genes involved in the PCP were down-regulated in Δ*xlnR*, but only on SBH in the *xkiA1* mutant compared to the wild type strain (Table S2 and Fig.S2). Interestingly, after 24h and 48h, the previously identified XlnR-target genes from the PCP, *xyrA* and *xdhA,* were down-regulated in both substrates at all time-points tested in Δ*xlnR*. XlnR seems to have a major influence on the expression of *xdhA* on both CS and SBH because in absence of XlnR, we do not observe the antagonistic interaction of AraR in regulation of this gene. None of the other PCP genes (*larA*, *ladA*, *lxrA* and *xkiA*) were consistently down-regulated in Δ*xlnR* (Table S2 and Fig.S2), but sometimes up-regulated at certain late time-points on CS or SBH, which implies that these genes are dependant on both XlnR and AraR on these crude substrates, but with a dominant regulatory role of AraR for the genes involved in the first three steps of te L-arabinose pathway. In Δ*xlnR, araR* was up-regulated after 4 and 24h of transfer to CS or SBH, compared to the wild-type strain (Table S3). These results correlate well with the previously reported antagonistic interaction of these regulators in *A. niger*, where it was shown that deletion of *xlnR* results in up-regulation of the PCP genes under control of AraR [5].

In the *xkiA1* mutant *araR* was up-regulated after 24h and 48h of transfer to CS, but only after 4h of transfer to SBH (Table S3). L-arabitol is the inducer from AraR and accumulates in the *xkiA1* mutant during growth on D-xylose or L-arabinose [29]. After 4h on both CS and SBH *xyrA* and *xdhA* were down-regulated, and similar results were observed for the extracellular enzymes releasing D-xylose residues. After 24 and 48h, transcript levels of these genes were not consistently down-regulated as observed in the *xlnR* deletion mutant. In contrast, transcript levels of *larA*, *ladA*, *lxrA* and *xkiA* on CS were up-regulated at some of the time points, while this was observed only for *ladA* and *lxrA* on SBH (Table S2 and Fig.S2). The results in our study indicate that conversion of pentoses and subsequent accumulation of L-arabitol and D-xylose in the *xkiA1* mutant might occur earlier in SBH than in CS. L-arabitol and D-xylose accumulation would cause up-regulation of the XlnR regulated genes at the early time-point on CS and SBH according to our hypothesis. However, the transcript levels of genes involved in the PCP and especially the extracellular response (xylanolytic and xyloglucan-active enzymes) appears to be similar to the *xlnR* deletion mutant after 4h. We hypothesize that L-arabitol or D-xylose might not have accumulated to a high enough level that it can (hyper) induce the XlnR regulated genes as it has been observed previously during growth on D-xylose and L-arabinose [13, 29].

As the PCP and PPP are interconnected, we also evaluated expression of genes involved in the PPP. Several genes involved in the PPP were down-regulated in Δ*xlnR*, after transfer to CS or SBH (Table S2). As expected, *talB*, previously identified as XlnR regulated gene, was down-regulated in both substrates at all the time points tested [36]. However, the other suggested XlnR-regulated gene (*rpiA*) was only down-regulated after 24h of transfer to SBH and therefore our results do not conclusively confirm that *rpiA* is only regulated by XlnR. Similarly, none of the other PPP genes were consistently down-regulated at all time points in Δ*xlnR*, which implies that they are not directly regulated by XlnR, but more likely indirectly affected to a different extent at the various time points.

L-rhamnose responsive regulator (RhaR)

RhaR controls expression of genes involved in RG-I degradation, as well as the L-rhamnose catabolic genes L-rhamnose-1-dehydrogenase (*lraA*), L-rhamnono-γ-lactonase (*lraB*) and L-rhamnonate dehydratase (*lraC*) during growth on L-rhamnose in *A. niger* [40-42]. Interestingly, *rhaR* was up-regulated at all the time points tested in SBH in Δ*xlnR*, as were *lraA*, *lraB* and *lraC* (Table S2-3 and Fig. S3). This may indicate that *A. niger* uses RhaR to (partially) compensate for the loss of XlnR. Such a compensation effect has recently been shown in *A. nidulans* between GalR, XlnR and AraR [43], and previously in *A. niger* for XlnR and AraR [5]. The RG-I main chain is cleaved by endo- (RHG) and exo-rhamnogalacturonase (RGX), unsaturated rhamnogalacturonan hydrolase (URGH), α-rhamnosidase (RHA) and rhamnogalacturonan lyase (RGL), with the assistance of rhamnogalacturonan acetyl esterase (RGAE) [1, 44, 45]. In our study, up-regulation of a number of RG-I degrading-enzymes was observed after 4h (eight enzymes) and 24h (thirteen enzymes) of transfer to SBH, correlating well with the up-regulation of RhaR. However, after 48h of growth the majority of pectinolytic genes involved in RG-I degradation were down-regulated.
Since in CS the amount of L-rhamnose is lower than in SBH, this pathway will not substantially contribute to growth on CS. Indeed, *lraA*, *lraB* and *lraC* were down-regulated after 24h and 48h of transfer to CS in the Δ*xlnR* strain. However, *rhaR* was only down-regulated after 48h of transfer to CS in Δ*xlnR*. In the *xkiA1* mutant, *rhaR* was up-regulated after 4h of transfer to SBH and down-regulated after 48h of transfer to CS (Table S3). These results correlate with the up-regulation of *lraA*, *lraB,* at all the time points tested, and *lraC* after 4h and 24h of transfer to SBH. In the *xkiA1* mutant on CS the results showed up-regulation of *lraA* and *lraC* after 4h and down-regulation of *lraA*, *lraB* after 24h and 48h, and *lraC* after 48h (Table S2 and Fig. S3). The up-regulation of *rhaR* after 4h of transfer to SBH might be sufficient to up-regulate the pathway genes at all time points. This up-regulation of *rhaR* correlates with the pectinolytic transcript levels. Five out of seven pectinolytic genes were involved in RG-I degradation and up-regulated after 4h on SBH in the *xkiA1* mutant. On CS, the down-regulation of *lraA*, *lraB* and *lraC* after 24h or 48h, correlated with the down-regulation of the majority of the pectinolytic genes at all the time points.

D-galacturonic-acid-responsive regulators: GaaR and GaaX

GaaR is a transcription factor required for growth on D-galacturonic acid and for the activation of the D-galacturonic acid responsive genes in *A. niger*. GaaX has been recently described as a repressor, inhibiting the transcription activity of GaaR under non-inducing conditions [46]. The majority of the GaaR-regulated genes encode enzymes needed for the degradation of homogalacturonan (HG), such as exo-polygalacturonases (PGX), endo-polygalacturonases (PGA), pectin methyl esterases (PME) and pectin lyases (PEL) [46]. Also, GaaR is required for induction of D-galacturonic acid reductase (*gaaA*), L-galactonic acid dehydratase (*gaaB*), 2-keto-3-deoxy-L-galactonate aldolase (*gaaC*) and L-glyceraldehyde/L-arabinose reductase (*gaaD*/*larA*) genes involved in D-galacturonic acid catabolism in *A. niger* [47]. After 4h of transfer to CS or SBH *gaaX* was down-regulated in Δ*xlnR* suggesting that the repression of GaaR by GaaX is removed in the absence of XlnR (Table S3). However, after 24h and 48h of transfer to SBH and 48h of transfer to CS *gaaX* was up-regulated in Δ*xlnR*, indicating that removal of repression is only an initial effect in this strain on CS. All genes involved in the D-galacturonic-acid metabolism were down-regulated in both substrates after 4h of transfer to CS or SBH in Δ*xlnR*. In the *xkiA1* mutant this was only the case for SBH. After 24h and 48h of transfer to SBH nearly all D-galacturonic acid pathway genes were up-regulated in both Δ*xlnR* and the *xkiA1* mutant (Additional file 2). The exception was *gaaD*/*larA*, which was not differentially expressed in the *xkiA1* mutant. After 24h on CS *gaaA* and *gaaB* were up-regulated in the *xkiA1* mutant, while *gaaD* was only up-regulated in Δ*xlnR.* After 48h on CS all genes were down-regulated in both deletion mutants. Expression of *gaaR* was not affected by *xlnR* deletion or *xkiA1* mutation on SBH at most of the time points tested. However, *gaaR* was down-regulated after 4h of transfer to CS in Δ*xlnR* and after 24h in the *xkiA1* mutant (Table S3). The down-regulation of *gaaR* might be due to other factors at the early time point and not due to a direct effect of *xlnR* deletion in Δ*xlnR* in CS.
The higher content of D-galacturonic acid present in SBH compared to CS likely explains the up-regulation observed after 24h and 48h of the first three genes involved in the pathway, while on CS all the pathway genes were down-regulated after 48h. On SBH, these results correlates with the up-regulation of several HG-degrading enzymes after 24h and 48h, while on CS the majority of the genes involved in the HG degradation were down-regulated at all the time points tested, in both deletion mutants.

The amylolytic regulator AmyR

AmyR is a transcriptional regulator that controls the genes involved in starch degradation, and it has been the first well-studied regulator in several *Aspergillus* species such as *A. nidulans* and *Aspergillus oryzae* [48, 49]. Expression of *amyR* was down-regulated at all time points in Δ*xlnR* grown on CS, and after 4h and 48h of transfer to CS in the *xkiA1* mutant (Table S3). These results correlate with the down-regulation of a number of starch degrading-enzymes after 4 and 48h of transfer to CS in the *xkiA1* mutant (Fig.3; Table S1 and Fig. S1).

After 4h of transfer to CS in the *xkiA1* mutant nine starch-degrading enzymes were down-regulated: *glaA*, six AGD genes (*agdA*, *agdB*, *agdC*, *agdD*, *agdE* and *agdF*) and two AMY genes (*aamA* and NRRL3_07699). After 48h of transfer to CS seven starch-degrading enzymes were down-regulated in the *xkiA1* mutant, *glaA* and six AGD genes (*agdA*, *agdB*, *agdC*, *agdD* and *agdE*).
In SBH *amyR* was only down-regulated after 24h in Δ*xlnR*, and after 4h in the *xkiA1* mutant. The down-regulation of *amyR* in the *xkiA1* mutant might be part of the initial response of *A. niger* after 4h of transfer to CS. In Δ*xlnR*, the results did not correlate with the expression of genes encoding starch-degrading enzymes in both substrates, suggesting an indirect effect of XlnR.

The cellulose regulators ClrA and ClrB

ClrA and ClrB are two TFs involved in the regulation of cellulose degradation, which have been partially characterized in *A. niger* [27]. It was shown that the interaction of two TFs, ClrB and McmA, is necessary for the regulation of *eglA* and *eglB* in *A. nidulans* [50], while in *A. niger*, expression of *cbhA*, *eglC* and *xynA* was shown to be affected by both XlnR and ClrB [49]. Expression of *clrA* was not affected on SBH at any time point tested in either of the deletion mutants. In contrast, *clrB* was down-regulated after 48h of transfer to SBH in Δ*xlnR*, and up-regulated after 24h and 48h of transfer to SBH in the *xkiA1* mutant (Table S3). In CS, *clrA* was down-regulated at all the time points tested in both deletion mutants, as was *clrB* after 4h in the *xkiA1* mutant and after 24h and 48h in Δ*xlnR*. These results indicate that ClrA or ClrB do not appear to compensate for the absence of XlnR, as observed previously in wheat straw [47]. The role of the homologs of these regulators (Clr1 and Clr2) has been studied in more detail in *Neurospora crassa*, where they are important regulators of genes encoding enzymes that are required for the degradation of cellulose. In contrast, the *N. crassa* XlnR homolog was not necessary for cellulase gene expression or activity [51], demonstrating diverse organization of the regulatory network in fungi. Clr1 and Clr2 appear to be essential in the cellulose degradation in *N. crassa*, but not in *A. niger* where XlnR is the major TF involved in the cellulose and hemicellulose degradation. At this point, no indications for a role of ClrA or ClrB in sugar catabolism have been reported and also our results do not suggest that they affect the expression profiles of the sugar catabolic genes.

In conclusion, in nature fungi are confronted with mixtures of carbon sources, and therefore likely activate a combination of the gene sets that were observed in response to crude substrates. Our understanding of the hierarchy of the transcriptional regulators and their interaction is still in its infancy, but appears to differ between fungal species. Our results also demonstrate that metabolic and regulatory mutations that result in a similar phenotype on pure sugars can cause significantly different physiology on crude substrates, especially after prolonged exposure.

The results of this study confirm that XlnR is the major regulator affecting expression of genes encoding (hemi-)cellulolytic enzymes in *A. niger*, but its influence appears to be dependent on the composition of the available substrates. This composition also strongly affects expression of CAZy genes that are not controlled by XlnR, such as those encoding pectin-degrading enzymes.

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Supplementary material

Table S1 | Expression of selected CAZymes involved in the degradation of plant biomass in *A. niger*. The comparisons between strains are Δ*xlnR* over the wild-type and *xkiA1* over the wild-type. The cut-off for differential expression is fold change >0.6 (cells marked red if up-regulated) and fold change <-0,6 (cells marked green if down-regulated) and p-value <0.05(cells marked yellow). **Available upon request from the author.**

Table S2 | Expression of known genes involved in central carbon metabolism in *A. niger*. The comparisons between strains are Δ*xlnR* over the wild-type and *xkiA1* over the wild-type. The cut-off for differential expression is fold change >0.6 (cells marked red if up-regulated) and fold change <-0.6 (cells marked green if down-regulated) and p-value <0.05(cells marked yellow). **Available upon request from the author.**

Table S3 | Expression of known regulators genes involved in plant biomass degradation in *A. niger*. The comparisons are between deletion mutants over the wild-type. The cutoff for differential expression if up-regulated fold-change >0.6 (cells marked dark grey) and fold-change <-0.6 if down-regulated (cells marked light grey) and p-value <0.05 (*).

[a]Abbrv.	Gene number	Δ*xlnR*/wt	*xkiA1*/wt	Δ*xlnR*/wt	*xkiA1*/wt
		4h-CS		4h-SBH	
XlnR	NRRL3_04034	-5.1*	-0.82*	-2.2*	0.16
AraR	NRRL3_07564	0.84*	0.25	1.4*	0.73*
RhaR	NRRL3_01496	-0.47	-0.15	1.9*	1.4*
GaaR	NRRL3_08195	-1.4*	-0.24	-0.36*	-0.072
GaaX	NRRL3_08194	-1.5*	-0.28	-2.2*	-1.4*
AmyR	NRRL3_07701	-0.7*	-1*	-0.27	-0.69*
ClrA	NRRL3_03544	-1.7*	-1.7*	0.045	-0.46*
ClrB	NRRL3_09050	-4.5*	-2.2*	-1.9*	-0.8*
CreA	NRRL3_05946	-1.3*	0.86*	-1.4*	0.0063
		24h-CS		24h-SBH	
XlnR	NRRL3_04034	-2.9*	0.097	-2.8*	0.42*
AraR	NRRL3_07564	0.9*	0.89*	1.2*	0.5*
RhaR	NRRL3_01496	-0.4	-0.25	0.93*	0.41
GaaR	NRRL3_08195	0.12	-0.63*	0.53*	0.33*
GaaX	NRRL3_08194	0.43*	-0.48*	1.1*	0.36
AmyR	NRRL3_07701	-2.1*	0.22	0.69*	0.13
ClrA	NRRL3_03544	-2.7*	-1.3*	0.43*	0.17
ClrB	NRRL3_09050	-2.1*	0.17	-0.3	1.1*
CreA	NRRL3_05946	2.2*	0.77*	-1*	-1.1*
		48h-CS		48h-SBH	
XlnR	NRRL3_04034	-2.6*	0.55*	-2.7*	0.26
AraR	NRRL3_07564	0.36*	0.92*	-0.091	-0.15
RhaR	NRRL3_01496	-0.66*	-1.4*	0.65*	-0.4
GaaR	NRRL3_08195	0.25	-0.24	0.35*	0.24
GaaX	NRRL3_08194	0.94*	0.39*	1.1*	0.39*
AmyR	NRRL3_07701	-2.7*	-0.9*	-0.24	-0.45*
ClrA	NRRL3_03544	-2.2*	-0.94*	0.084	0.15
ClrB	NRRL3_09050	-1*	1.3*	-0.87*	0.94*
CreA	NRRL3_05946	2.9*	2.2*	-2*	-0.71*

[a]List of regulators genes based on Benocci et *al.,* 2017 [54].

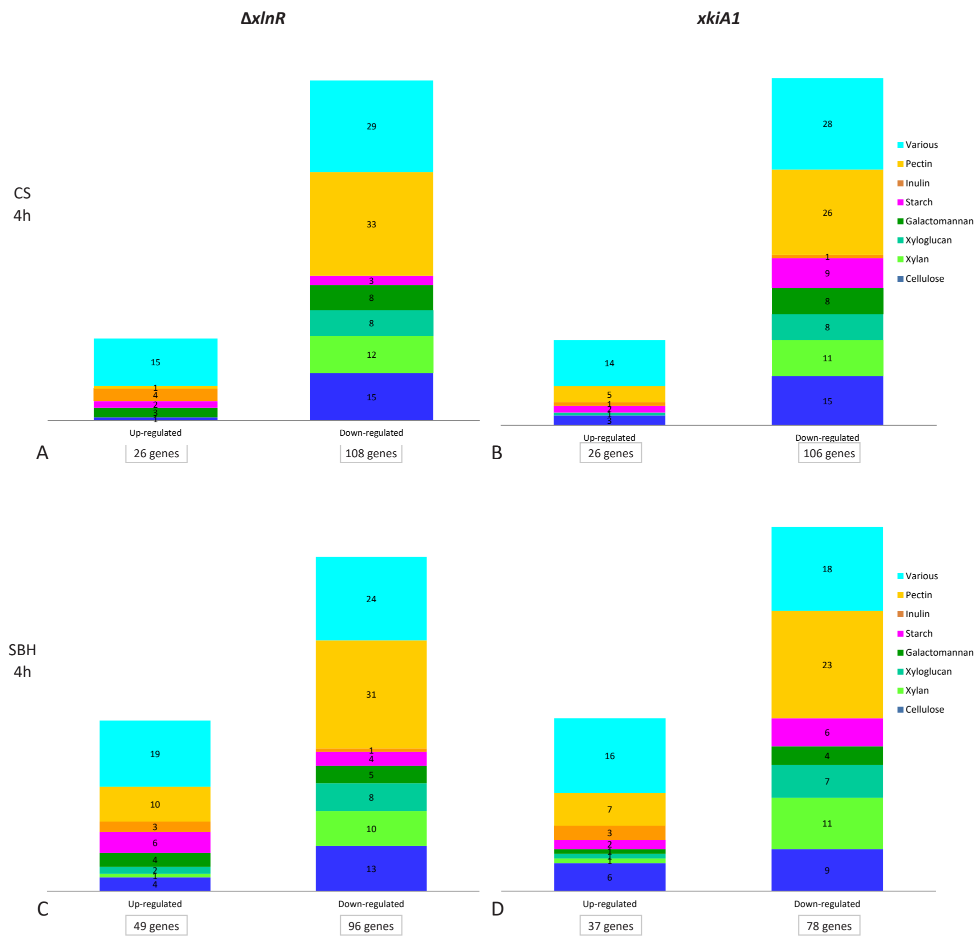

Figure S1 | CAZy genes involved in the degradation of plant biomass in *A. niger* that are significantly up-regulated or down-regulated between Δ*xlnR* vs the wild-type (A;C) and between *xkiA1* vs the wild-type (B;D) after 4h of transfer to CS (A;B) and SBH (C;D). The gene numbers are listed in Table S1.

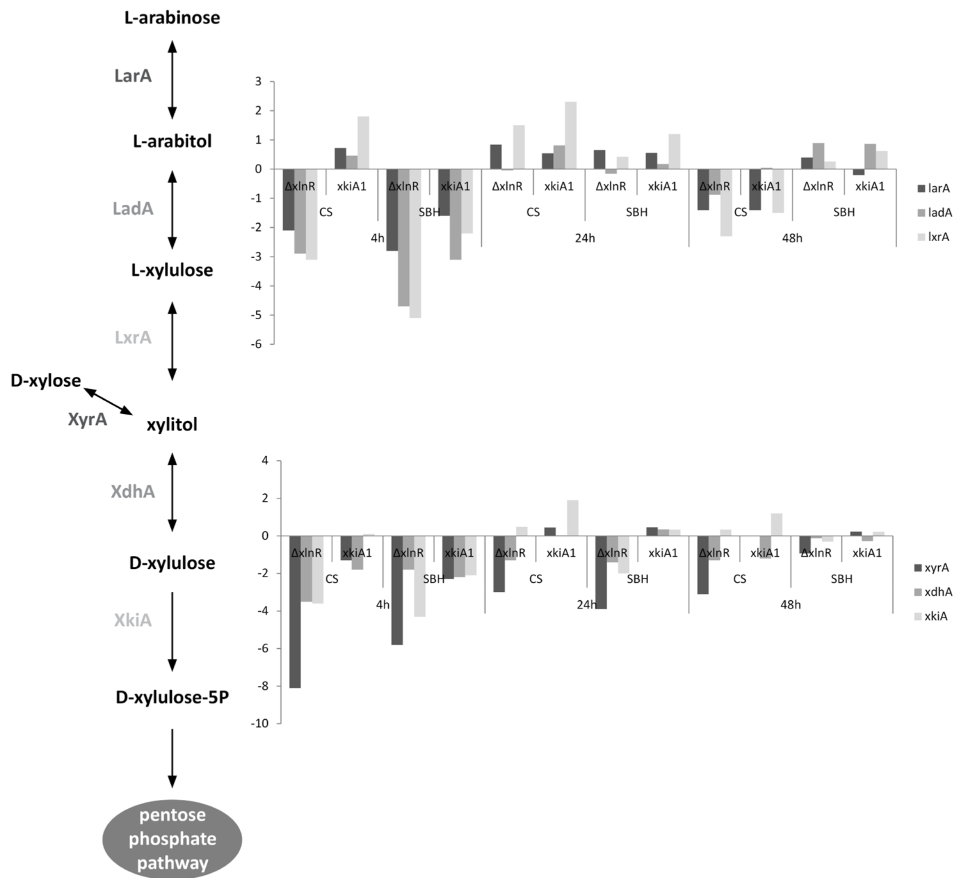

Figure S2 | Representation of pentose catabolic pathway, including expression profiles of the genes involved in the pathway.

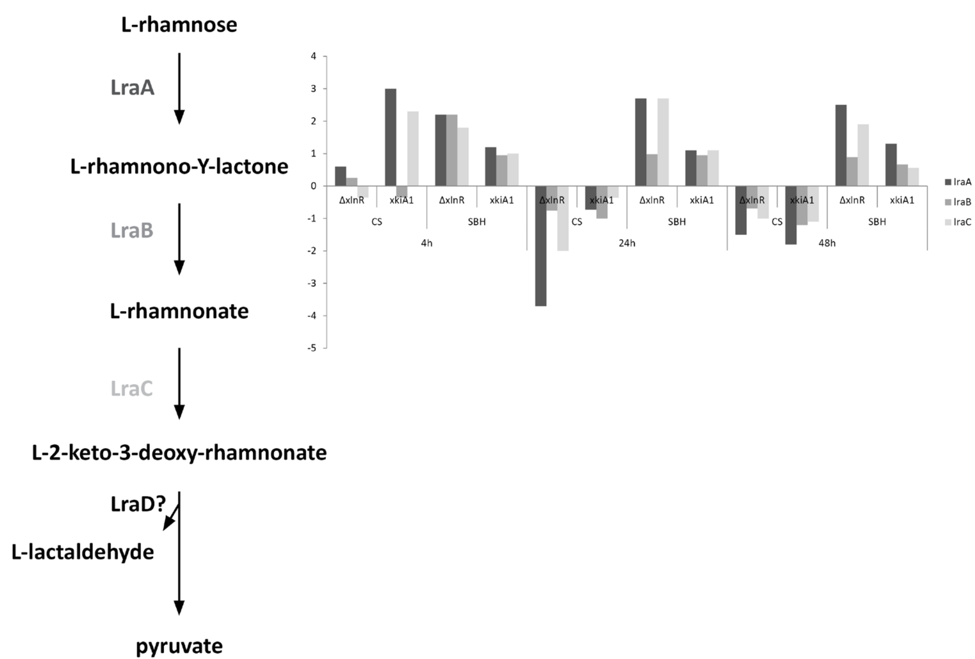

Figure S3 | Representation of L-rhamnose pathway, including expression profiles of the genes involved in the pathway.

Chapter 5

High resolution visualization and exo-proteomics reveal the physiological role of XlnR and AraR in plant biomass colonization and degradation by *Aspergillus niger*

Kowalczyk JE*, Khosravi C*, Purvine S, Dohnalkova A, Chrisler WB, Orr G, Robinson E, Zink E, Wiebenga A, Peng M, Battaglia E, Baker SE, and de Vries RP; *Environ. Microbiol,* 10.1111: 1462-2920.

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Abstract

In *Aspergillus niger*, two transcription factors, AraR and XlnR, regulate production of enzymes involved in degradation of arabinoxylan and catabolism of the released L-arabinose and D-xylose. Deletion of both *araR* and *xlnR* leads to reduced production of (hemi)cellulolytic enzymes and reduced growth on arabinan, arabinogalactan and xylan. In this study we investigated the colonization and degradation of unprocessed wheat bran by the *A. niger* reference strain CBS 137562 and *araR*/*xlnR* regulatory mutants using high-resolution microscopy and exo-proteomics. This enabled us to link the reduction in colonization and degradation efficiency of the mutants to the absence of subsets of enzymes. This study for the first time visualizes the correlation between enzyme production and substrate colonization and reveals which enzymes play an essential role in this process.

Introduction

Saprobic fungi have a great impact on the environment because of their ability to decompose dead organic matter. *Aspergillus niger*, found globally in nearly all biotopes, produces a wide range of extra-cellular enzymes which synergistically degrade plant polymers found in the environment. As a cosmopolitan fungus, *A. niger* faces diverse biopolymers and therefore has to adjust its enzyme production so that this optimally matches the available substrates. The more complex the plant biomass is, the more complex the arsenal of enzymes needs to be that the fungus produces. Wheat bran (WB) is the outer layer of wheat (*Triticum aestivum*) grain, separated from the starchy endosperm during wheat milling into flour. It contains mainly cell wall polysaccharides, including cellulose, (arabino)xylan and xyloglucan, but also significant amounts of starch, lignin, mineral compounds and proteins [1]. When growing on WB, *A. niger* secretes an array of Carbohydrate Active enZymes (CAZymes) necessary for production of monomeric nutrients [2]. The expression and secretion of CAZymes by *A. niger* exposed to complex biomass, has been reported to be sequential [3-5]. For example, Borin et al. (2015) [3] showed that xylan and pectin-degrading enzymes were secreted earlier than cellulose-degrading enzymes in *A. niger* grown on steam-exploded sugarcane bagasse. To avoid wasting energy on the production of enzymes that are not needed, the composition of the produced enzyme mixture must be tightly regulated at the transcriptional level [6].

Transcription factors (TFs) are mediators of transcriptional regulation, the primary mechanism by which fungi control gene expression. Action of TFs depends on the presence (and concentration) of simple mono/oligomeric signal molecules that are released from the environment by low level constitutively expressed or starvation-influenced scouting enzymes [4, 7]. XlnR, the (hemi-)cellulolytic master regulator in *A. niger*, controls the expression of genes encoding xylan-degrading β-endoxylanases (*xlnB*, *xlnC*), β-xylosidase (*xlnD*) [8, 9], α-glucuronidase (*aguA*), acetyl xylan esterase (*axeA*), arabinoxylan arabinofuranohydrolase (*axhA*), feruloyl esterase (*faeA*) [9-11], α-galactosidase (*aglB*) and β-galactosidase (*lacA*) [12] as well as cellulose-degrading β-endoglucanases (*eglA*, *eglB*, *eglC*) [9] and cellobiohydrolases (*cbhA*, *cbhB*) [13].

The arabinanolytic regulator AraR, a close homolog of XlnR, controls expression of genes encoding α-arabinofuranosidases (*abfA*, *abfB*) [14]. Deleting both TFs in *A. niger* leads to incomplete degradation of D-xylose and L-arabinose containing polysaccharides, such as xylan, arabinan, arabinogalactan and apple pectin [14]. However, a Δ*araR*Δ*xlnR* strain is still able to grow on WB suggesting that the regulation of CAZyme production in response to crude substrates is much more intricate, than can be explained by the action of XlnR and AraR. A previous study [15] showed differences in gene regulation by AraR and XlnR in *A. niger* when simple monomeric sugars (L-arabinose or D-xylose), mixed monomeric sugars (L-arabinose with D-xylose) or complex crude biomass (sugarcane bagasse) were used in the growth medium.

During the saccharification of sugarcane bagasse, both TFs were essential for the expression of the pentose catabolic pathway genes but XlnR had a larger influence on the expression of genes encoding plant biomass degrading enzymes [15].
The role of XlnR and AraR in regulation of enzyme production has been extensively studied during growth on both simple and complex sugars using transcriptomics. However, colonization of natural substrates by *A. niger* at the microscope level and in particular which effect these TFs have in the ability of *A. niger* to colonize crude plant biomass, has never been addressed.
In this study, we examined the colonization and degradation of WB by *A. niger* using high-resolution microscopy and exo-proteomics. Moreover, we compared the results of the reference strain to mutants in which one (Δ*araR* or Δ*xlnR*) or two TFs (Δ*araR*Δ*xlnR*) have been deleted.

Materials and Methods

Strains and growth conditions

All *A. niger* strains used in this study are listed in Table 1. The strains were maintained at 30°C on plates containing solidified (1.5% agar) Complete Medium [16] with 1% glucose. For auxotrophic strains, the media were supplemented with 1 mg L^{-1} nicotinamide, 0.2 g L^{-1} leucine, 0.2 g L^{-1} arginine or 1.22 g L^{-1} uridine. Fresh conidia were harvested with ACES buffer and diluted in Minimal Medium [16] without any carbon source to the final concentration of 10^6 conidia/ml. Dried, unprocessed WB was obtained from a mill “de Vlijt” (Wageningen, the Netherland). WB monosaccharide composition was 42 mol% glucose, 34.6 mol% xylose, 16.5 mol% arabinose, 3.3 mol% uronic acid, 1.7 mol% galactose and 1.4 mol% mannose [2]. For solid state fermentations, 10 ml of spore suspension was mixed with 5 g of dry autoclaved, crude WB in a Petri dish and incubated at 30°C. The samples of mycelium-WB aggregates were taken 20 and 40 hpi (hours past inoculation) for microscopic and secretome analysis. All samples were collected and analyzed in triplicate. The time points were chosen based on the germination progress observed under the binocular (Nikon SMZ1000) and represent the early and late stage of wheat bran colonization.

Microscopy sample preparation

Hyphal penetrations were analyzed by Helium Ion (HIM) and Confocal Laser Scanning (CLSM) microscopy. For HIM, the mycelium–wheat bran samples were preserved in 2.5% glutaraldehyde, washed three times in PBS and dehydrated in an ethanol series (33 %, 50 %, 75 %), followed by three washes in 100 % ethanol. The samples were then critically point dried in a CPD instrument (Samdri-795; Tousimis) and processed according to an automated CPD scheme, with CO_2 as a transitional fluid. The cells were mounted on standard aluminum HIM stubs covered with double-sided carbon adhesive tape, and sputter-coated with carbon.

Table 1 | The *A. niger* strains used in this study.

Strain	CBS number	Genotype	Reference
Reference strain	CBS 137562 (NW249)	*cspA1, nicA1, leuA1, ΔargB, pyrA6*	[17]
ΔaraR	CBS 141249	*cspA1, nicA1, leuA1, pyrA6, ΔargB::pIM2101(argB+), ΔaraR*	[14]
ΔxlnR	CBS 141248	*cspA1, nicA1, leuA1, ΔargB, pyrA6::A. oryzae pyrG, ΔxlnR*	[14]
ΔaraRΔxlnR	CBS 141250	*cspA1, nicA1, leuA1, ΔargB::pIM2101(argB+), ΔaraR, pyrA6::A. oryzae pyrG, ΔxlnR*	[14]

For CLSM, the samples were fixated for 20 min in PBS buffered 4% paraformaldehyde solution, followed by three 5 min washing steps with PBS. The paraformaldehyde fixed fungal biofilm was stained for 60 minutes with 10 µg/ml propidium iodide (ThermoFisher) for plant cells and 20 min with 5 µg/ml FM 1-43 (ThermoFisher) to visualize fungal structures and in PBS buffer (Sigma). Samples were then washed with PBS, placed in a 35-mm culture plate and covered by the buffer. Images were acquired at 1.81 mm z-steps on a Zeiss LSM 710 scanning head confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) with a Zeiss W Plan-Apochromat 20x/1.0 DIC M27 75mm objective. Excitation lasers were 458 and 561 nm for the green and red emission channels, respectively. FM 1-43 was detected at 510-597 nm and propidium iodide was detected at 627-719 nm. Laser dwell times were 0.79 ms for both channels. Image analysis (2D and 3D) was conducted using Volocity (Perkin Elmer). Images were processed to determine penetration of WB by the fungal hyphae.

Protein extraction and digestion

For secretome analysis, the mycelium-wheat bran samples were mixed with 10 ml of cold distilled water and incubated for 1h with gentle shaking. The samples were then centrifuged for 10 min at 3220 x *g* and supernatants which contained proteins secreted by the strains were collected. The proteins were extracted using a standard TCA precipitation method and digested as described previously, with modifications [18]. Briefly, the protein pellets were dissolved in 8M urea in 50 mM NH_4HCO_3 (pH 8.0) with 1% CHAPS, then DTT was added to a final concentration of 5 mM and the proteins were denatured and reduced at 60°C for 30 min in a thermomixer. Protein solutions were then diluted 10-fold with 50 mM NH_4HCO_3 (pH 8.0) and $CaCl_2$ was added at 1 mM concentration, followed by 3h digestion with Trypsin (USB) at an enzyme-to-protein ratio of 1:50. Salts and detergents were removed using an SCX SPE column (Supelco, St. Louis, MO). Before use, the columns were conditioned with 3 mL of MeOH and rinsed twice with 2mL of 0.1% and 2ml of 1% TFA (trifluoroacetic acid) solution. The peptide solutions were slowly loaded onto the column (not faster than 1 mL/minute), washed with 4 mL of 70% methanol in 0.1% TFA in water and eluted with 1 mL of 5% NH_4OH in 30% methanol in water.

Peptides were concentrated to dryness, reconstituted to 100 µL in nanopure water and centrifuged at 10 000 x *g* for 5 min to remove any particulates. The protein concentration was estimated using a BCA protein assay and the samples were diluted to 0.1 µg μL^{-1} for LC-MS/MS processing.

LC-MS/MS mass spectrometry analysis

All data were collected on hybrid Velos linear ion trap coupled Orbitrap mass spectrometers (Thermo Electron, Waltham, MA, USA) coupled to Waters NanoAcquity or Next-Gen 3 high performance liquid chromatography systems (Waters Corporation, Milford, MA, USA) through 75 µm x 70 cm columns packed with Phenomenex Jupiter C-18 derivatized 3 µm silica beads (Phenomenex, Torrance, CA, USA). Samples were loaded onto columns with 0.05% formic acid in water and eluted with 0.05% formic acid in acetonitrile over 100 minutes. Ten data-dependent MS/MS scans were recorded for each survey MS scan using normalized collision energy of 35, isolation width of 2.00, and rolling exclusion window of +1.55/-0.55 Th lasting 60 seconds before previously fragmented signals are eligible for re-analysis.

MS/MS data searching

The MS/MS spectra from all LC-MS/MS datasets were converted to ASCII text (.dta format) using DeconMSn [19] which more precisely assigns the charge and parent mass values to an MS/MS spectrum. The data files were then interrogated via target-decoy approach [20] using MSGFPlus [21] with a +/- 50 ppm parent mass tolerance, no specific digestion enzyme settings, and a variable posttranslational modification of oxidized methionine. Proteins were obtained from the Joint Genome Institute (http://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html) using the annotation developed by A. Tsang and co-workers, resulting in 11,839 non-redundant protein entries. These were coupled with common contaminant protein sequences such as albumin, keratins, and tryptic fragments.

Accurate Mass and Time (AMT) tag relative quantitation

The peptide abundance values were quantitated as described previously [22], briefly following these steps: Collating MS/MS results into a relational database (Microsoft SQL Server), extracting unique peptide sequence exact IUPAC masses and relative normalized elution times (NETs) [23]; extracting LC-MS elution peaks; warping LC-MS scan numbers to relative normalized elution times; recalibrating mass error; matching LC-MS peaks to the unique peptide masses and NETs calculated earlier using VIPER [24]; storing resultant masses and their respective proteins to the relational database; exporting the peptide abundances for each sample involved, along with attendant protein level information, to Excel.

Label Free quantitation post processing

Peptide abundances were log(2) linearized and normalized using mean central tendency in Inferno (https://omics.pnl.gov/software/infernordn) [25], followed by abundance summing to protein level. Protein abundances were log(2) linearized, log differences normalized to 0, and protein level Z-Scores reported for samples being compared. Proteins exhibiting Z-Scores greater than 2 were considered significant and worthy of further study. The experimental exo-proteome was filtered to only include proteins that are predicted to be secreted by the *A. niger* NRRL3 strain and was based on the secretion peptide prediction available at http://genome.jgi.doe.gov. The enzymes were categorized according to the Carbohydrate Active Enzymes database (CAZy) classification available at http://www.cazy.org/_ENREF_30 [26].

Results

Unprocessed WB has a smooth and rough surface

Colonization of WB by the *A. niger* reference strain CBS 137562 and the AraR and XlnR regulatory mutants was visualized using Helium Ion (HIM) and Confocal Laser Scanning (CLSM) Microscopy. Two time points, 20 and 40 hours post inoculation (hpi), were compared as they represent an early and late stage of substrate colonization. Crude, unprocessed WB was used to visualize the fungus feeding on the substrate as it would in nature. During the analysis, differences in the appearance of uncolonized WB flakes were observed. One side of the flake had a smooth appearance, with a relatively firm and lined texture (Fig. 1). This side of the flake was commonly convex in shape and in this study we refer to it as “smooth surface”. The other side of the WB flake had an uneven appearance, often decorated with circular residues (Fig. 1). This side of the flake was commonly concave in shape and we refer to it as “rough surface”. These differences are most likely caused by mechanical separation of the outer bran and the inner endosperm during milling.

A. niger strains in which *xlnR* is deleted are not able to colonize the smooth surface of WB

In the early stage of substrate colonization (20 hpi), the hyphae of the reference strain were growing on both the smooth and rough surface of WB (Fig. S1 A and B). The Δ*araR* mutant colonized the rough surface of WB but no hyphae were detected on the smooth side (Fig. S1 C and D), while the Δ*xlnR* strain grew very poorly on the rough surface and no visible colonization was observed on the smooth surface (Fig. S1 E and F). The double mutant Δ*araR*Δ*xlnR* was only colonizing the rough surface of WB and localized mostly near the cut edges and cracks in the surface of WB (Fig. S1 G and H).

In the late stage of WB colonization (40 hpi), the hyphae of the reference strain were growing abundantly on both sides of WB, occasionally penetrating the substrate (Fig. 2 A and B, Movie S1). Matured conidiophores were observed on the rough side of the flake.

The Δ*araR* mutant grew abundantly on the rough surface, where matured conidiophores were spotted (Fig. 2 C and D). Some growth was observed on the smooth surface, mostly near the edges of the flake. At 40 hpi, colonization of WB by Δ*xlnR* or Δ*araR*Δ*xlnR* was similar. Both strains were slightly colonizing the rough side of WB and near cut edges and cracks of the WB particle, but were absent on the smooth side (Fig. 2 E, F, G and H, Movie S2).

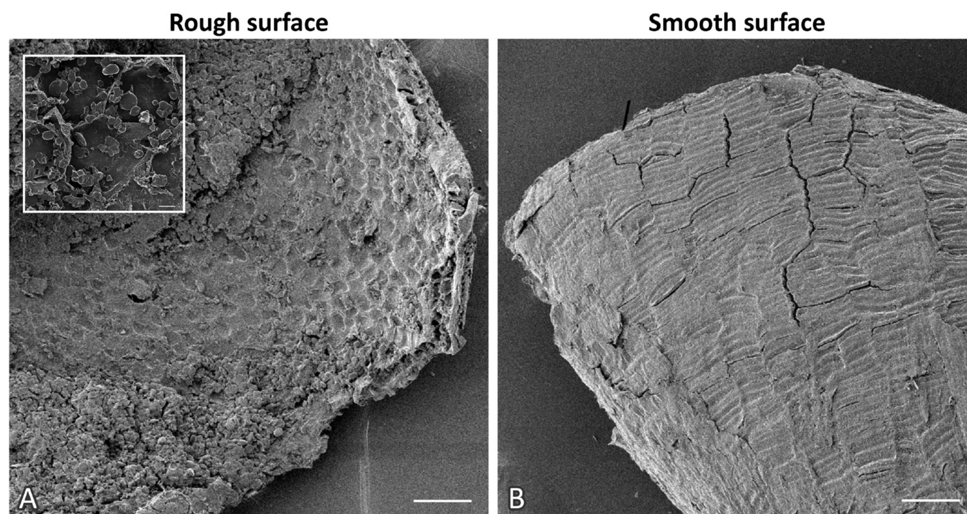


Figure 1 | The rough (A) and smooth (B) side of the WB flake. The images were taken with HIM.

The *araR* and *xlnR* deletions affect the secretome size in *A. niger*

The exo-proteome of the *A. niger* reference strain and the regulatory mutants grown on WB was analyzed to explain the differences in colonization abilities among the strains. All experimentally identified proteins were qualified (number of detected proteins) and quantified (protein abundance). A complete list of proteins secreted by the reference strain, Δ*araR*, Δ*xlnR* and Δ*araR*Δ*xlnR* mutants is presented in Table S1.

Only the highly confident secreted proteins which have been identified in the exo-proteome and contain a predicted signal sequence (based on SignalP information from http://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html) were used for further comparison. The regulatory mutants secreted fewer proteins than the reference strain at both time points tested. During the early stage of WB colonization (20 hpi), the reference strain secreted 313 proteins, 120 of which were identified as CAZymes (Fig. 3, Table S1). Δ*araR* secreted 216 proteins, of which 81 (33% less than the reference) were identified as CAZymes. Δ*xlnR* secreted 256 proteins, with 115 (4% less than the reference) classified as CAZymes. Δ*araR*Δ*xlnR* secreted 230 proteins, of which 85 (29% less than the reference) were identified as CAZymes.

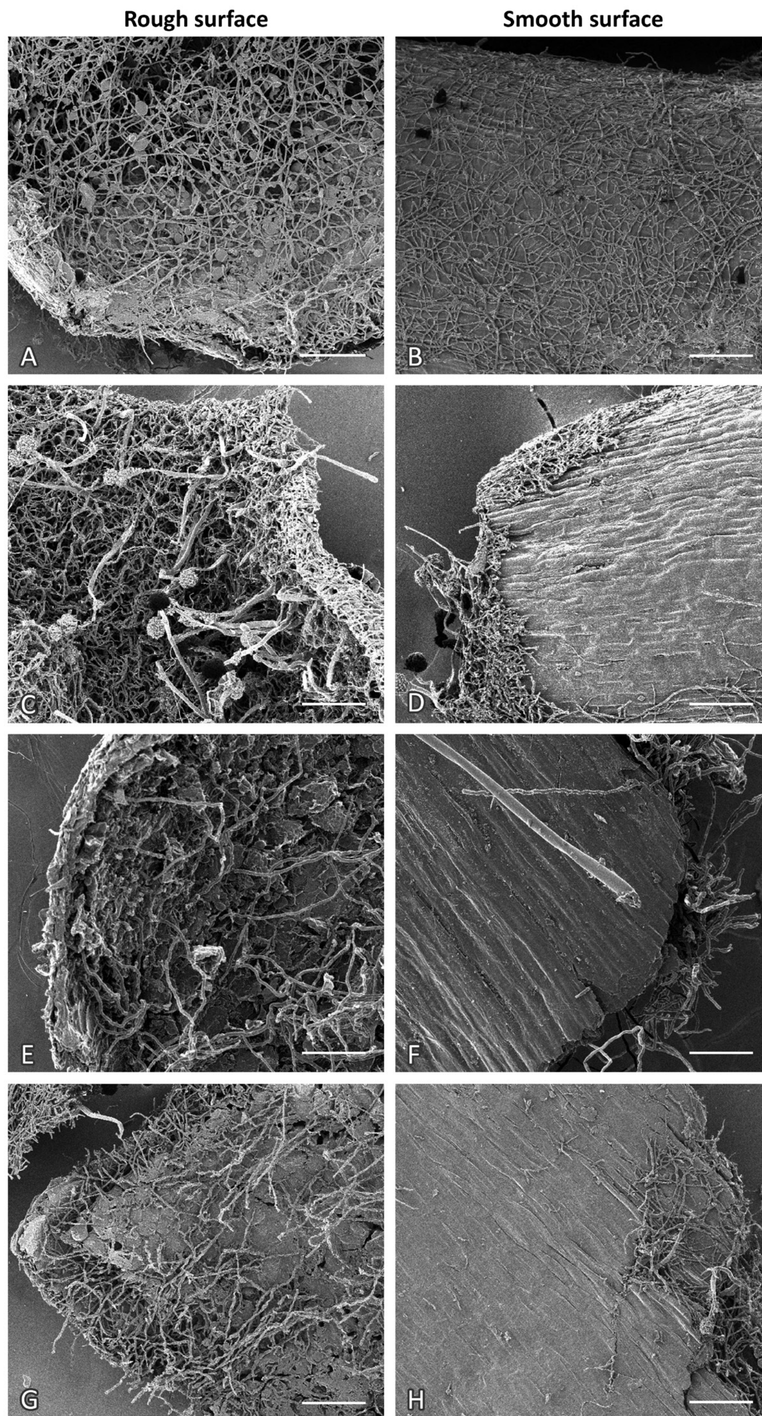
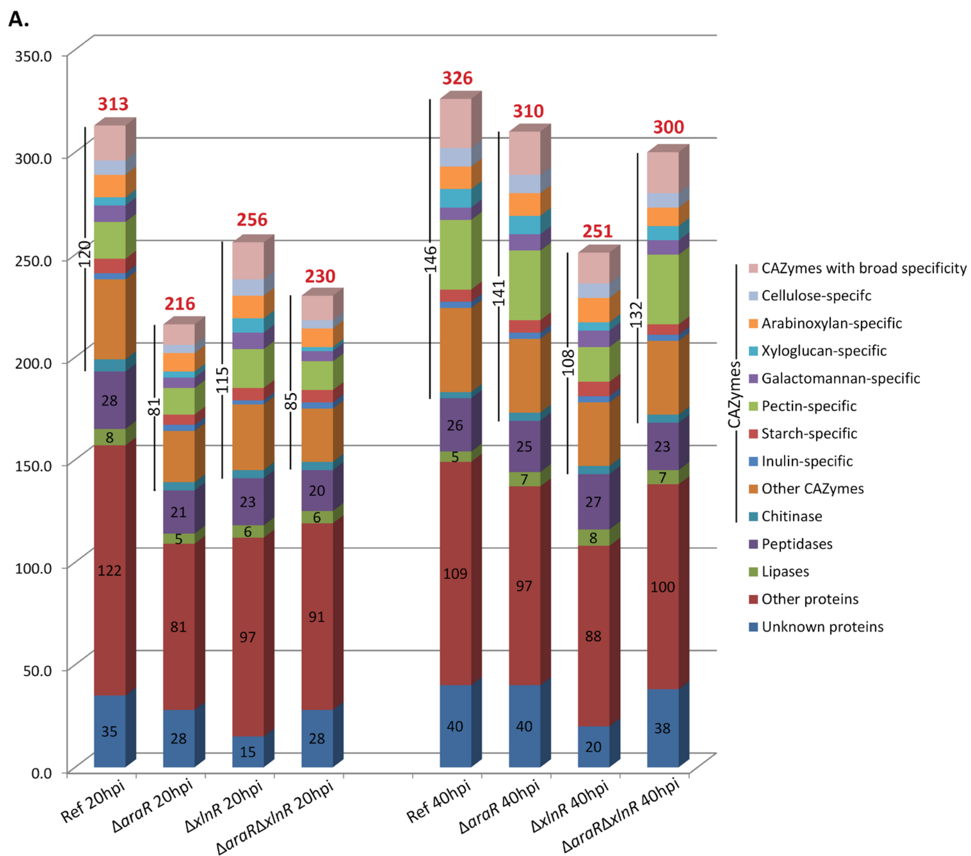


Figure 2 | Colonization of WB by the *Aspergillus niger* reference strain and regulatory mutants observed 40 hpi. The images capture the growth of the reference strain (A, B); Δ*araR* (C, D), Δ*xlnR* (F, G) and Δ*araR*Δ*xlnR* (H, I) on the rough and smooth surface of WB and were taken with HIM. The scale bars represent 100 µm.

During the late stage of WB colonization (40 hpi), the reference strain secreted 326 proteins, and 146 of them were identified as CAZymes (Fig. 3, Table S2). Δ*araR* secreted 310 proteins, of which 141 (3% less than the reference) were identified as CAZymes. Δ*xlnR* secreted 251 proteins, of which 108 (26% less than the reference) were CAZymes. The Δ*araR*Δ*xlnR* secreted 300 proteins. 132 (10% less than the reference) of them were identified as CAZymes. Longer incubation time of *A. niger* on WB resulted in higher amounts of secreted proteins in all strains tested, except for Δ*xlnR*, which secreted roughly the same number of proteins in both time points tested.



B.

	Δ*araR*	Δ*xlnR*	Δ*araR*Δ*xlnR*
20 hpi	-33%	-4%	-29%
40 hpi	-3%	-26%	-10%

Figure 3 | A) Overview of proteins identified in the secretome of the reference strain, Δ*araR*, Δ*xlnR* and Δ*araR*Δ*xlnR* grown on WB for 20 and 40h. B) Percentage of secreted CAZymes that are absent in this mutant compared to the reference.

Deletion of *araR* and *xlnR* resulted in reduced abundance of (hemi)cellulolytic enzymes

The (hemi)cellulose-active CAZymes were of particular interest due to the high amount of cellulose and arabinoxylan in WB. These enzymes were divided into five sub-groups based on substrate specificity: cellulose-specific, arabinoxylan- specific, galactomannan- specific, xyloglucan-specific and CAZymes with broad specificity (Fig. 3, Table S1). The cellulose-specific sub-group included β-1,4-endoglucanase (EGL), cellobiohydrolase (CBH) and cellobiose dehydrogenase (CDH). At 20 and 40 hpi, we found for the reference strain seven and nine secreted cellulose-specific enzymes, respectively. During the early colonization stage (20 hpi), two putative CBHs (NRRL3_10870, NRRL3_09019) and one EGL (EglC) were not present in the secretome of Δ*araR* and Δ*araR*Δ*xlnR* (Table 2). The Δ*xlnR* strain produced the same enzymes as the reference strain, with one additional CBH (CbhB).

During the late stage of WB colonization (40 hpi), one EGL (EglA) and one putative CBH (NRRL3_09019) were not secreted in the strains in which *xlnR* was deleted (Table 2). Additionally, the abundance of CbhA was reduced in Δ*xlnR* and another putative CBH (NRRL3_10870) was reduced in Δ*araR*Δ*xlnR*.

The arabinoxylan-specific sub-group comprised of β-1,4-endoxylanases (XLN), β-1,4-xylosidases (BXL), arabinoxylan arabinofuranohydrolase (AXH), α-glucuronidase (AGU) and acetyl xylan esterases (AXE). All regulatory mutants had a strongly reduced ability to produce arabinoxylanolytic enzymes in the early stage of WB colonization (20 hpi). The abundance of one XLN (XynA), two BXLs (XlnD, GbgA) and AguA was reduced or not detected in Δ*araR* when compared to the reference strain (Table 2). In Δ*xlnR*, the abundance of two XLNs (XynA, XynV) was reduced. The strongest effect was observed in Δ*araR*Δ*xlnR*, where the abundance of XynA, XynV, XlnD, AxhA and AxeA was reduced, while GbgA and AguA were not detected in the secretome.

In the late stage of WB decay (40 hpi), only the strains in which *xlnR* was deleted had a reduced ability to produce arabinoxylan-specific enzymes. The abundance of one putative BXL (NRRL3_10881), AguA and Axe was reduced or not detected in Δ*xlnR* and Δ*araR*Δ*xlnR*, while two BXLs (XlnD and XarB) were not secreted in Δ*araR*Δ*xlnR* (Table 3). The galactomannan-specific sub-group contained β-1,4-endomannanases (MAN), β-1,4-mannosidases (MND) and α-1,4-galactosidases (AGL). During early colonization (20 hpi), the abundance of one putative MAN (NRRL3_08912), one MND (MndA) and three AGLs (AglA, AglB, AglC) was either reduced or not detected at all in all strains in which *araR* was deleted (Table 3). The Δ*xlnR* mutant had reduced abundance of one putative MAN (NRRL3_04196) and one AGL (AglC). Interestingly, increased abundance of AglA and AglB were detected in Δ*xlnR* after 20 h on WB, while the abundance of the same enzymes was reduced after 40 h. The xyloglucan-specific sub-group included 1,4-endoglucanases (XEG), α-xylosidases (AXL) and α-fucosidases (AFC). During the early colonization stage, one XEG (EglA) and one AXL (AxlA) were either reduced or not detected in both Δ*araR* and Δ*araR*Δ*xlnR*, while one putative AFC (NRRL3_01396) was not present in any of the regulatory mutants (Table 3).

Table 2 | Abundance of cellulose-specific CAZymes secreted over time by the *A. niger* reference strain and regulatory mutants on WB. The abundance is the averaged and logged (base of 2) value of three replicates. Only proteins present in at least two out of three replicates were considered. Empty position means that the protein was not produced under the conditions tested or not detected using our experimental settings. Statistical significance was calculated using the 2 sigma method. Significantly reduced abundance in the mutant compared to the reference strain is marked by light grey shading and a star behind the number. If the protein was absent in the mutant when compared to the reference strain, the change was also considered significant and marked by light gray shading and a star.

Substrate	Protein ID	Protein name	CAZy family	Ref 20 h	Δ*araR* 20 h	Δ*xlnR* 20 h	Δ*araR* Δ*xlnR* 20 h	Ref 40 h	Δ*araR* 40 h	Δ*xlnR* 40 h	Δ*araR* Δ*xlnR* 40 h
Cellulose	NRRL3_02585	EglA	GH5					31.0	28.9	*	*
	NRRL3_06791	EglB	GH5	36.1	36.0	37.6	34.8	39.6	39.1	36.6	39.2
	NRRL3_04917	EglC	GH5	35.8	*	35.2	*	37.8	38.3	34.9	35.2
	NRRL3_11105	EGL	GH131	34.7	33.5	33.5	33.3	35.9	37.2	36.2	37.5
	NRRL3_10870	CBH	GH6	32.0	*	35.0	*	36.8	38.0	33.1	32.2*
	NRRL3_09019	CBH	GH6	31.1	*	30.8	*	33.2	35.6	*	*
	NRRL3_04953	CbhA	GH7	30.5	29.6	33.4	29.1	37.9	39.1	32.6*	39.1
	NRRL3_02584	CbhB	GH7			28.3		33.7	33.4	30.5	34.6
	NRRL3_06316	CDH	AA8,AA3	34.9	35.0	32.6	33.9	33.3	35.5	35.7	36.1

During the late stage of WB colonization, two putative XEGs (NRRL3_08380, NRRL3_01918) and three putative AFCs (NRRL3_01396, NRRL3_07382, NRRL3_07089) were not found in Δ*xlnR*, but only two of them (NRRL3_01918 and NRRL3_07382) were also not present in the double mutant. Additionally, the abundance of AxlA was reduced in the Δ*araR*Δ*xlnR* strain.

The last sub-group, CAZymes with broad specificity, consists of enzymes that act on various polysaccharides such as α-arabinofuranosidase (ABF), feruloyl esterase (FAE), β-1,4-galactosidase (LAC), β-1,4-glucosidase (BGL) and lytic polysaccharide monooxygenase (LPMO). These activities are necessary for complete depolymerization of cellulose, arabinoxylan and xyloglucan which are present in WB. The strains in which *araR* was deleted had reduced ability to secrete AbfA at both time points tested, while the abundance of AbfB was either reduced or not detected only in the early colonization stage (20 hpi) (Table 4). All FAEs secreted by the reference strain (FaeA, FaeB, FaeC) were reduced or not secreted in the Δ*araR* and Δ*araR*Δ*xlnR* strains during the early colonization stage. In Δ*xlnR,* the abundance of two FAEs (FaeA and FaeC) was reduced after 20 h, while FaeB was not produced after 40 h on WB. The abundance of two LACs (LacA, LacB) was reduced or not detected, respectively, in the *araR* single and double mutants during early colonization, while LacB and LacE were affected by both *araR* and *xlnR* deletion at 40 hpi (Table 4). Three putative BGLs were secreted by the reference strain at 20 h, of which two (NRRL3_10133, NRRL3_4818) were not detected in the Δ*araR* and Δ*araR*Δ*xlnR* strains. Five putative BGLs (NRRL3_07975, NRRL3_09976, NRRL3_03953, NRRL3_10133, NRRL3_09644) were not produced in the Δ*xlnR* strain, while one putative BGL (NRRL3_09644) was missing in Δ*araR* after 40 h on WB. Interestingly, abundance of other putative BGLs (NRRL3_03953, NRRL3_00742) increased in Δ*araR*Δ*xlnR* compared to the reference strain. The abundance of one putative LPMO (NRRL3_03929) was reduced in Δ*xlnR* during early colonization*,* while another (NRRL3_07568) was not detected in all tested mutants (Table 4). During the late stage of WB colonization, three putative LPMOs were missing in Δ*araR* (NRRL3_03961, NRRL3_08994, NRRL3_00415) and Δ*xlnR* (NRRL3_07568, NRRL3_03772, NRRL3_00415). Interestingly, the abundance of only two putative LPMOs (NRRL3_03961, NRRL3_08994) was reduced in the double mutant.

Substrate	Protein ID	Protein name	CAZy family	Ref 20 h	Δ*araR* 20 h	Δ*xlnR* 20 h	Δ*araR* Δ*xlnR* 20 h	Ref 40 h	Δ*araR* 40 h	Δ*xlnR* 40 h	Δ*araR* Δ*xlnR* 40 h
(Arabino) xylan	NRRL3_08708	XynA/XlnC	GH10	39.5	34.8*	34.2*	35.7*	37.9	40.7	39.1	38.7
	NRRL3_01648	XynB/XlnB	GH11	35.6	32.6	35.8	33.2	38.5	39.6	35.5	37.1
	NRRL3_02827	XynIV	GH11	31.6	30.8	30.7	30.4	33.9	35.0	34.1	35.3
	NRRL3_03928	XynV	GH11	35.8	32.4	31.0*	32.0*	33.0	34.8	34.9	34.5
	NRRL3_02451	XlnD/XynD	GH3	38.6	31.9*	37.1	30.6*	40.1	40.7	36.2	32.4*
	NRRL3_06419	XarB	GH3	31.9	31.6	31.1	31.8	34.5	33.4	31.1	28.2*
	NRRL3_10881	BXL	GH43	33.6	30.9	33.0	31.1	36.1	36.4	30.4*	*
	NRRL3_11773	GbgA	GH43	33.2	*	35.9	*	37.3	36.3	35.8	36.6
	NRRL3_09890	XynVI/XynD	GH43							28.0	
	NRRL3_08707	AxhA	GH62	38.8	35.6	39.6	35.3*	41.7	42.5	38.5	39.9
	NRRL3_01069	AguA	GH67	34.9	*	31.8	*	35.6	37.0	30.7*	*
	NRRL3_03339	AxeA	CE1	32.4	30.9	35.1	28.3*	38.2	39.1	33.0*	33.9*
Galacto-mannan	NRRL3_08912	MAN	GH5	30.5	*	31.6	*	34.9	38.3	36.0	38.3
	NRRL3_04196	MAN	GH26	36.8	35.1	31.0*	34.5		33.6	35.9	30.3
	NRRL3_09612	MndA	GH2	34.6	30.5*	34.3	30.1*	37.8	38.6	35.0	39.3
	NRRL3_11747	AglA	GH27	30.5	*	34.8*	*	36.5	33.7	31.6*	35.2
	NRRL3_05358	AglB	GH27	33.5	29.0*	37.3*	26.8*	39.2	39.3	34.2*	39.5
	NRRL3_00016	AglC	GH36	35.5	34.1	30.7*	34.7	31.3	35.4	34.5	36.6*
	NRRL3_00743	AglD	CBM35	31.7	*	33.7	*	36.1	33.5	32.3	31.2*
	NRRL3_09622	AGL	GH27	32.1	32.3	30.7	31.7		28.4	30.1	
Xyloglucan	NRRL3_00819	EglA	GH12	35.8	28.6*	38.1	29.1*	39.1	40.8	36.7	38.4
	NRRL3_08392	XEG	GH12	33.6	32.1	30.4	31.7	33.3	34.7	33.4	34.5
	NRRL3_08380	XEG	GH12					31.8	31.6	*	32.3
	NRRL3_01918	XEG	GH12			30.4		28.4	30.7	*	*
	NRRL3_01787	EglC	GH74			29.3		34.3	34.5	32.0	34.7
	NRRL3_00268	AxlA	GH31	34.6	28.3*	33.2	*	35.9	36.2	32.2	30.9*
	NRRL3_01396	AFC	GH29	28.6	*	*	*	29.5	30.1	*	30.6
	NRRL3_07382	AFC	GH95			32.8		35.6	34.2	*	*
	NRRL3_07089	AFC	GH95			30.2		33.1	31.5	*	30.0

Table 3 | Abundance of hemicellulose related CAZymes secreted over time by the *A. niger* reference strain and regulatory mutants on WB. The abundance is the averaged and logged (base of 2) value of three replicates. Only proteins present in at least two out of three replicates were considered. Empty position means that the protein was not produced under the conditions tested or not detected using our experimental settings. Statistical significance was calculated using the 2 sigma method. Significantly reduced abundance in the mutant compared to the reference strain is marked by light grey shading and a star behind the number. If the protein was absent in the mutant when compared to the reference strain, the change was also considered significant and marked by light gray shading and a star. Significantly increased abundance in the mutant compared to the reference strain is marked by dark gray shading and a star behind the number.

The presence of starch facilitates growth of the mutants on the rough side of crude WB

To gain more insight into the WB colonization strategy, ten proteins with the highest abundance in the reference strain were analyzed. In the early stage of WB colonization (20 hpi), arabinoxylan degrading enzymes XynA, AxhA, XylD and FaeA are among the most abundantly secreted in the reference strain (Table 4), while the abundance of these enzymes were reduced in Δ*araR*Δ*xlnR.* Additionally, the reference strain abundantly secreted two pectin-specific enzymes, PgaB and PelC, which were not affected by the tested TF deletions. In the late stage of WB colonization (40 hpi), a glucoamylase (GlaA) was the most abundantly secreted protein in the reference strain, followed by an α-amylase (AamA). Those CAZymes are involved in the depolymerisation of starch, the major plant storage polysaccharide. Production of amylolytic enzymes was not affected by *xlnR* and *araR* deletions. Therefore, the presence of starch on the rough side of WB would facilitate growth of the mutants.

Table 4 | Abundance of CAZymes with broad specificity secreted over time by the *A. niger* reference strain and regulatory mutants on WB. The abundance is the averaged and logged (base of 2) value of three replicates. Only proteins present in at least two out of three replicates were considered. Empty position means that the protein was not produced in the conditions tested or not detected using our experimental settings. Statistical significance was calculated using the 2 sigma method. Significantly reduced abundance in the mutant compared to the reference strain is marked by light grey shading and a star behind the number. If the protein was absent in the mutant when compared to the reference strain, the change was also considered significant and marked by light gray shading and a star. Significantly increased abundance in the mutant compared to the reference strain is marked by dark gray shading and a star behind the number.

Substrate	Protein ID	Protein name	CAZy family	Ref 20h	Δ*araR* 20h	Δ*xlnR* 20h	Δ*araR* Δ*xlnR* 20h	Ref 40h	Δ*araR* 40h	Δ*xlnR* 40h	Δ*araR* Δ*xlnR* 40h
Not specific	NRRL3_01606	AbfA	GH51	38.2	31.7*	37.7	30.9*	39.1	35.9*	37.3	34.6*
	NRRL3_03768	AbfB	GH54	37.2	*	37.0	29.6*	39.9	38.4	36.9	38.6
	NRRL3_00007	FaeA	CE1	38.5	30.4*	34.1*	30.6*	37.1	38.8	35.8	33.5*
	NRRL3_02931	FaeB	CE1	29.9	*	31.1	*	34.5	35.2	*	36.8
	NRRL3_08993	FaeC	CE1	35.2	*	29.1*	28.4*		28.7	33.2	
	NRRL3_11738	LacC	GH35	29.9	31.3	30.0	30.4	33.2	31.1	29.9	31.0
	NRRL3_01071	LacE	GH35					31.5	27.9*	*	29.7
	NRRL3_02630	LacA	GH35	35.1	29.5*	36.0	29.6*	38.2	36.8	34.7	37.6
	NRRL3_02479	LacB	GH35	31.7	*	28.2	*	33.0	29.6*	28.0*	31.8
	NRRL3_07975	BGL	GH1			26.3		29.7	29.0	*	28.0
	NRRL3_09976	BGL	GH1					29.5	28.0	*	*
	NRRL3_10449	Bgl1	GH3	36.2	32.8	35.0	33.4	38.6	39.3	34.9	38.8
	NRRL3_03953	BGL	GH3					25.8	28.7	*	31.7*
	NRRL3_10133	BGL	GH3	31.6	*	*	*	33.1	32.8	*	34.1
	NRRL3_00742	BGL	GH3			27.6		27.4	30.6	29.0	35.1*
	NRRL3_09644	BGL	GH3					26.7	*	*	26.9
	NRRL3_04818	BGL	GH3	28.9	*	30.8	*			31.0	
	NRRL3_03929	LPMO	AA9	36.4	33.9	31.6*	34.4	29.8	31.3	35.9	32.4
	NRRL3_11147	LPMO	AA9	36.3	35.6	33.8	35.5	35.7	38.6	37.2	39.3*
	NRRL3_03961	LPMO	AA9	36.2	36.1	34.2	34.9	29.1	*	37.3	*
	NRRL3_08994	LPMO	AA9	35.4	32.5	32.3	32.5	27.1	*	35.0	*
	NRRL3_00814	LPMO	AA9	33.1	30.9	29.5	31.4	34.1	35.8	33.1	35.9
	NRRL3_07568	LPMO	AA9	28.8	*	*	*	27.3	29.0	*	29.3
	NRRL3_03383	LPMO	AA9					30.4	27.5		

Table 5 | Ten of the most abundant proteins identified in the secretome of the *A. niger* reference strain grown on WB for 20 and 40h. The abundance shown in the Table is the averaged and logged (base of 2) value of three replicates. Only proteins present in two out of three replicates were considered. The proteins that were significantly reduced in the mutant compared to the reference strain are marked by grey shading and a star. The significance was calculated using the 2 sigma method.

	Protein ID	Protein name	Ref	Δ*araR*	Δ*xlnR*	Δ*araR* Δ*xlnR*
20 h	NRRL3_00987	aspartic peptidase, extracellular	40.6	38.1	40.2	38.4
	NRRL3_08708	endoxylanase XynA/XlnC	39.5	34.8*	34.2*	35.7*
	NRRL3_05859	endopolygalacturonase PgaB	38.9	38.3	37.5	38.1
	NRRL3_08707	arabinoxylan arabinofuranohydrolase AxhA	38.8	35.6	39.6	35.3*
	NRRL3_09811	pectin lyase PelC	38.8	38.9	36.7	38.0
	NRRL3_02451	β-xylosidase XylD/XynD	38.6	31.9*	37.1	30.6*
	NRRL3_00007	feruloyl esterase FaeA	38.5	30.4*	34.1	30.6*
	NRRL3_08300	glucoamylase GlaA	38.5	36.5	39.4	35.2*
	NRRL3_09843	FAD-binding domain-containing protein	38.4	38.2	37.9	38.4
	NRRL3_01606	α-arabinofuranosidase AbfA	38.2	31.7*	37.7	30.9*
40 h	NRRL3_08300	glucoamylase GlaA	43.0	41.2	38.6	43.9
	NRRL3_00987	aspartic peptidase, extracellular	42.9	42.1	39.8	41.7
	NRRL3_08707	arabinoxylan arabinofuranohydrolase AxhA	41.7	42.5	38.5	39.9
	NRRL3_09875	alpha-amylase AamA	41.1	38.9	36.6*	41.7
	NRRL3_02451	beta-xylosidase XylD/XynD	40.1	40.7	36.2	32.4*
	NRRL3_03768	alpha-N-arabinofuranosidase AbfB	39.9	38.4	36.9	38.6
	NRRL3_11091	prolyl endopeptidase EPR	39.6	38.1	35.0*	38.2
	NRRL3_06791	endoglucanase	39.6	39.1	36.6	39.2
	NRRL3_05358	alpha-galactosidase AglB	39.2	39.3	34.2*	39.5
	NRRL3_01606	alpha-N-arabinofuranosidase AbfA	39.1	35.9*	37.3	34.6*

Discussion

Many fungi can thrive in nature thanks to their ability to produce plant polysaccharide degrading enzymes. These extracellular enzymes provide carbon for the fungus by breaking down complex plant polymers into more accessible monomers. The strains in which the transcriptional activator genes *araR* and/or *xlnR* were deleted have a reduced ability to degrade plant biomass because AraR and XlnR regulate production of the majority of the (hemi)cellulolytic enzymes of *A. niger* [9, 14]. In this study we analyzed the colonization of unprocessed wheat bran (WB) by the *A. niger* reference strain CBS 137562, a derivative of *A. niger* N402, and of the Δ*araR,* Δ*xlnR* and double Δ*araR*Δ*xlnR* mutants using high-resolution microscopy and exo-proteomics. WB is a by-product of the milling industry and is one of model substrates to study natural responses of *A. niger* to plant biomass.

Our data showed that WB flakes have a "rough" and a "smooth" surface (Fig. 1) with different affinity towards fungal hyphae. The smooth side is the outside of the wheat kernel and can be considered to be the intact part of the bran. The rough side represents the inside of the wheat bran that was connected to the endosperm, and was disrupted during milling. This could therefore also be considered as partially broken part of the biomass.
Our results clearly indicate that colonization of the smooth side of WB is more difficult for the regulatory mutants than colonization of the rough side. During the early stage of colonization (20 hpi), all strains in which *araR* and *xlnR* were deleted were not able to grow on the smooth surface of WB. Previous reports indicated that xylanolytic enzymes are secreted before cellulolytic enzymes by *A. niger* grown on complex substrates [3]. This correlates with our data, which indicates that arabinoxylan-active enzymes are among the most abundant proteins secreted during early stage of WB colonization by the reference strain (Table 5). After 20 h of growth on WB, the abundance of several important XLNs, ABFs, BXLs and FAEs were reduced in the *araR* and *xlnR* mutants, with the strongest effect observed in Δ*araR*Δ*xlnR* (Table 3). Absence and/or reduction of the abundance of those enzymes might explain the impaired colonization of *araR* and *xlnR* missing strains on the smooth surface of WB. During the late colonization stage, only strains in which *xlnR* was deleted could not colonize the smooth surface of WB (Figs 2 and S1). This could be linked to the absence/reduction of several cellulolytic (EglA, NRRL3_09019) and arabinoxylanolytic enzymes (NRRL3_10881, AguA, AxeA) in the Δ*xlnR* single and double mutant compared to the reference strain (Tables 1 and 2). Production of these enzymes was not affected in the *araR* deletion mutant and this strain could still partially colonize the smooth side of WB after 40 h (Fig. 2). These results also indicate that XlnR is the major regulator that drives colonization of WB in *A. niger*, and that other regulators, e.g. ClrA/B [6], cannot compensate for the loss of this regulator.

Growth on the rough side of WB was observed regardless of the regulatory mutation. This suggests that the rough side of WB contains other polysaccharides, such as starch, that can support growth of the strains. The presence of starch-degrading enzymes was detected in the reference strain when grown on WB for 40 hpi (Table 5). Moreover, the abundance of these enzymes was high in the regulatory mutants during growth on WB (Table S1). Starch is a storage polysaccharide that naturally occurs in wheat, but mainly localizes in the endosperm. Our data suggest that during WB production, some starch-rich residues of endosperm cells are deposited on the rough side of WB, creating an area that is relatively easy to degrade by the regulatory mutants, as their mutations do not affect the production of starch-degrading enzymes.

Biofilm formation during growth of *A. niger* on solid substrates is affected by its ability to produce plant polysaccharide degrading enzymes but also depends on other factors such as surface interactions.

Hydrophobins are versatile surface-active proteins involved in reduction of water surface tension, conidiation, attachment to hydrophobic surfaces and enzyme recruitment among others [27-29]. A recent study showed that two hydrophobins, RodA and DewC, contribute to biofilm formation in *A. nidulans* grown on sugarcane bagasse [30]. The Hyp1 protein, homolog of RodA in *A. niger*, was secreted in the reference strain grown on WB for 40 h but was absent in Δ*xlnR* (Table S1b). Lack of Hyp1 might contribute to reduced growth observed in Δ*xlnR* by (1) weakening mycelium adhesion and biofilm formation and (2) reducing enzyme recruitment and retention. It is unclear at this point whether expression of *hyp1* is XlnR-dependent in *A. niger*. However, transcriptome data showed that *hyp1* is highly expressed on lignocellulose [4] and contains three XlnR binding sites in its promoter region, which might suggest direct regulation.

In conclusion, our data demonstrate the physiological role of two major transcriptional regulators, XlnR and AraR, in plant biomass colonization and degradation by *A. niger*. XlnR is the major regulator required for this process and loss of this regulator impaired the fungus in colonization of wheat bran particles.

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Supplementary material

Movie S1 | Late stage of WB colonization by *A. niger* reference strain observed by CLSM. The substrate is colored in red and the fungal hyphae are green. **Available upon request from the author.**

Movie S2 | Late stage of WB colonization by *A. niger* Δ*araR*Δ*xlnR* observed by CLSM. The substrate is colored in red and the fungal hyphae are green. **Available upon request from the author.**

Table S1 | A complete list of proteins identified in the secretome of *A. niger* reference strain and regulatory mutants after 20 and 40 h of growth on WB. **Available upon request from the author.**

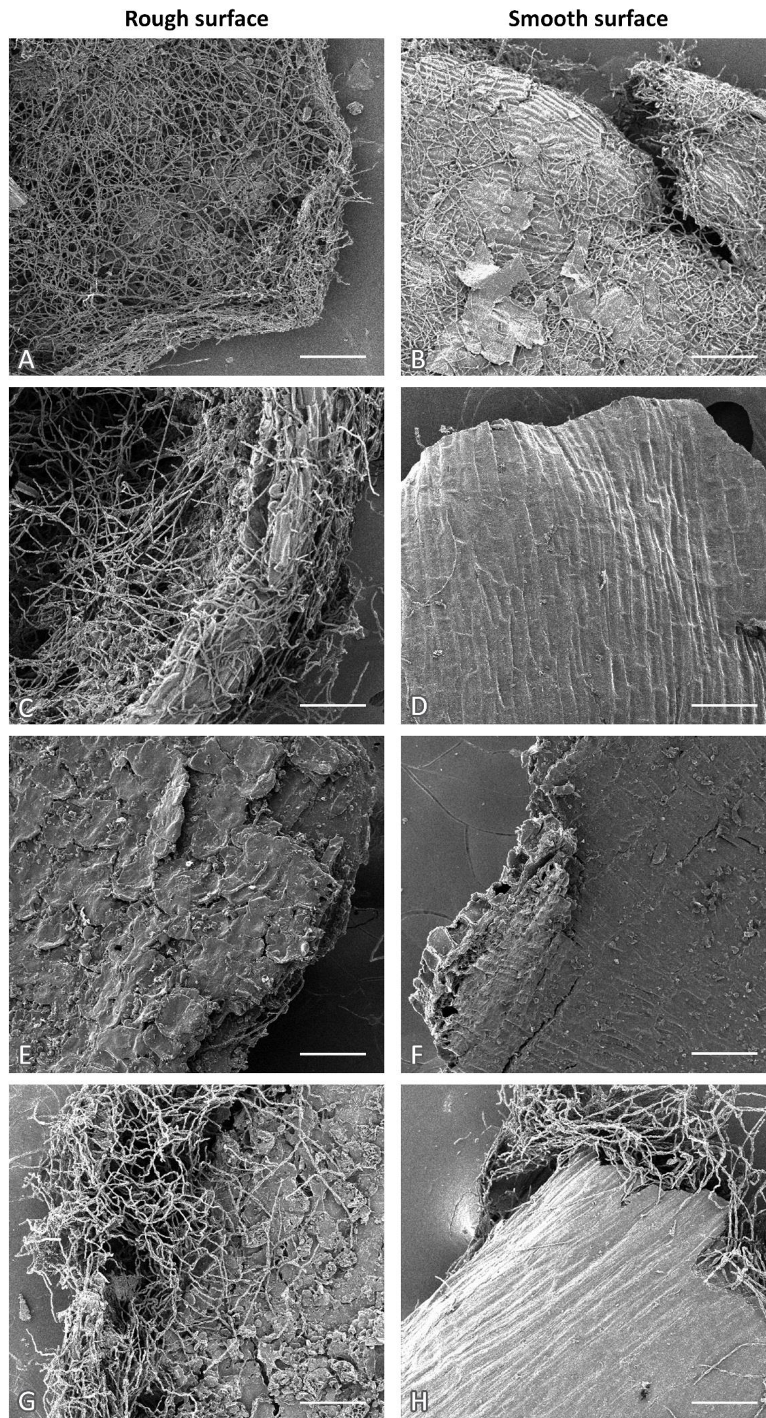

Figure S1 | Colonization of WB by *A. niger* reference strain and regulatory mutants observed 20 hpi. The pictures capture the growth of the reference strain (A, B); Δ*araR* (C, D), Δ*xlnR* (F, G) and Δ*araR*Δ*xlnR* (H, I) on rough and smooth surface of WB and were taken with HIM. The scale bars represent 100µm.

Chapter 6

Re-routing central carbon metabolism in *Aspergillus nidulans*

Khosravi C, Battaglia E, Dalhuijsen S, Visser J, Aguilar-Pontes MV, Zhou MM, Heyman HM, Kim YM, Baker SE, de Vries RP- under review in *BMC Genomics* (2017)

Abstract

Plant biomass is the most abundant carbon source for many fungal species. In the biobased industry fungi are used to produce of lignocellulolytic enzymes to degrade agricultural waste biomass. Here we evaluated if it would be possible to create an *Aspergillus nidulans* strain that releases but does not metabolize hexoses from plant biomass. For this purpose, metabolic mutants were generated that were impaired in glycolysis, by using hexokinase (*hxkA*) and glucokinase (*glkA*) negative strains. To prevent repression of enzyme production due to the hexose accumulation, strains were generated that combined these mutations with a *creAΔ4* mutant, the repressor involved in regulating preferential use of different carbon catabolic pathways. Phenotypic analysis revealed reduced growth for the *hxkA1 glkA4* mutant on wheat bran. However, hexoses did not accumulate during growth of the mutants on wheat bran, suggesting that glucose metabolism is re-routed towards alternative carbon catabolic pathways. The *creAΔ4* in combination with preventing initial phosphorylation in glycolysis resulted in an increased expression of pentose catabolic and pentose phosphate pathway genes. This indicates that the reduced ability to use hexoses as carbon sources created a shift towards the pentose fraction of wheat bran as a major carbon source to support growth.

Introduction

Plant biomass is the main renewable material on earth and the major starting material for several industrial processes. In nature, *Aspergillus* degrades plant biomass polysaccharides to obtain monomeric sugars that can serve as a carbon source. *Aspergillus* is able to secrete enzymes that can hydrolyse polysaccharides into pentoses and hexoses [1], which can be taken up by the fungus. *Aspergillus* then uses a variety of catabolic pathways to efficiently convert all monomeric components of plant biomass. D-glucose is a hexose that can be phosphorylated to glucose-6-phosphate by either glucokinase (GlkA) [2] or hexokinase (HxkA) [3] in *Aspergillus nidulans* (Fig. 1). Glucose-6-phosphate can then either enter the pentose phosphate pathway (PPP) or can be converted to fructose-6-phosphate by phosphoglucose isomerase (SwoM) [4] and enter glycolysis. D-fructose is phosphorylated by hexokinase (HxkA) to fructose-6-phosphate, which can enter glycolysis [3, 5] selected hexokinase and glucokinase mutants based on their resistance to 2-deoxy-D-glucose (2DOG) in the presence of 1% (v/v) glycerol as carbon source. These mutants were used in our study. The critical step in bio-ethanol production is the release of sugars from plant material, because the enzymes used in this process are expensive to produce and purify. A possibility to optimize the process is to create a fungal strain that can hydrolyse the polymeric fraction but cannot utilize fermentable sugars such as D-glucose and D-fructose. With such a strain the plant degrading enzymes do not have to be produced and purified in a separate environment, but the fungus itself can be used to generate these sugars during growth on plant biomass. Such a 'one-pot' bioethanol process has been reported by combining cellulase production and ethanol production using a co-cultivation of *Acremonium cellulolyticus* and *Saccharomyces cerevisiae* [6] . Also, others studies in which *S. cerevisiae* was co-cultured with other fungi revealed an increase in ethanol production and a reduction of the fermentation time and cost [7].

The genomes of most filamentous fungi contain all the necessary glycolytic genes for conversion of D-glucose and D-fructose. The aim of this study was firstly to combine the carbon catabolite derepressed strain with a glucose and fructose non-consuming strain, redirecting metabolism towards consumption of other sugars (e.g. pentoses). Secondly, to analyze the effect of these strains on a complex substrate. Previous phenotypic analysis of glucokinase and hexokinase mutants from *A. nidulans* [5] and *Aspergillus fumigatus* [8] indicated that HxkA and GlkA are the main catalytic hexose kinases [3]. At least one of them is required for normal growth on D-fructose or D-glucose. It has been shown that growth of the *A. fumigatus* Δ*hxkA* Δ*glkA* strain is reduced on D-glucose and D-fructose [8]. The *A. nidulans hxkA1 glkA1* mutant has been shown to be unable to phosphorylate D-glucose and D-fructose [5]. Phosphorylation of hexose sugars by both glucokinase and hexokinase was shown to play a role in mediating CCR via CreA, but it was not essential.

Furthermore, genes involved in ethanol utilization, xylan degradation and acetate catabolism were derepressed in this *A. nidulans glkA4 hxkA1* mutant [5]. However, de-repression of these genes was not complete suggesting CCR via CreA is mediated via other mechanisms triggered by substrate-specific signals.

In order to redirect consumption in the hexokinase/glucokinase mutant and overcome carbon catabolic repression via CreA, in this study we created a strain in which a progeny of the *creAΔ4* described by Shroff et *al.,* 1997 [9] is combined with glucokinase and hexokinase negative traits. The resulting mutant strains have been analysed on wheat bran with regard to growth, extra - and intracellular sugar accumulation, extracellular enzyme production, and gene expression.

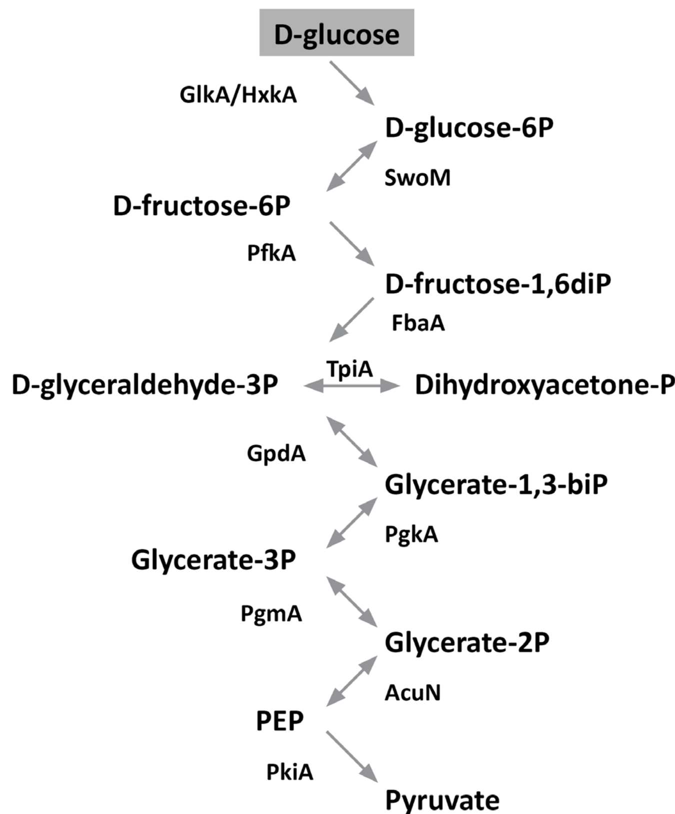

Figure 1 | Schematic representation of glycolysis in *A. nidulans*.
GlkA4: glucokinase (AN8689); HxkA1: hexokinase (AN7459); SwoM: phosphoglucoisomerase (AN6037); PfkA: phosphofructokinase (AN3223); FbaA: fructose-1,6-biphosphate (AN2875); TpiA: triose-P-isomerase (AN6900); GpdA: glyceraldehyde-3-phosphate dehydrogenase (AN8041); PgkA: phosphoglycerokinase (AN1246); PgmA: phosphoglycerate mutase (AN3059); AcuN: enolase (AN5740); PkiA: pyruvate kinase (AN5210).

Materials and Methods

Strains, media and growth conditions

All *A. nidulans* strains used in this study are listed in the Table 1 and were grown in minimal medium (MM) or complete medium (CM) with addition of a carbon source [18].

Table 1 | *A. nidulans* strains used in this study. (In bold are the strains that were used in all the experiments).

Name	CBS accession number	Genotype	References
UUAM101.08	**CBS 141343**	***y*A2**	**This study**
UUAM104.47	**CBS 141344**	***creAΔ4*, *pyro*A4**	**This study**
UUAM102.37	**CBS 141756**	***hxkA1 glkA4*, *yA2*, *paba*A1, *pyro*A4**	**This study**
UUAM104.87	**CBS 141447**	***creAΔ4 hxkA1 glkA4, y*A2, *pyro*A4**	**This study**
UUAM101.24		*y*A2, *pyro*A4	This study
UUAM102.71		*hxkA1 glkA4, y*A2, *pyro*A4	This study
NW299		*glkA4*, *y*A2, *pyro*A4	[5]
NW193		*hxkA1 glkA4, paba*A1	[5]
V148		*creAΔ4*, *panto*A1	[9]

The nitrogen source used in our media is sodium nitrate (6g/l). For all the analyses performed, two biological replicates were used, except for the metabolomics where three biological replicates were used. For the growth on plates, 1.5% (w/v) agar was added to the medium. For the production of spores, the strains were cultivated on plates containing CM supplemented with 1% xylose and incubated at 37°C in the dark for 5 days. The spores were harvested in ACES buffer and counted with haemocytometer (THOMA) under 40x magnifications. Square plates of MM plus 1% polysaccharide or 3% crude carbon source were used for growth profile. When necessary, the medium was supplemented with pyridoxine (pyro, 0.2 $g.L^{-1}$), pantothenic acid (panto, 0.1 $g.L^{-1}$) and para-aminobenzoic acid (paba, 0.2 $g.L^{-1}$). The plates were inoculated with 2 µl spore solutions at $5x10^5$ spores/ml and incubated at 37°C during 3-4 days. For the transfer experiments, all the strains were pre-grown in 1 L Erlenmeyer flasks that contained 200 ml of CM plus 2% D-xylose. Liquid cultures were inoculated with 10^6 spores/ml and shaken at 250 rpm in a rotary shaker. After 16 h, the mycelium was harvested by filtration, washed twice with MM and transferred to 250 ml Erlenmeyer flaks containing 50 ml MM plus 1% wheat bran (w/v) and the appropriate supplements, at 37°C. The mycelium was harvested by vacuum filtration and culture samples were taken after 2, 8 and 24 h of incubation. The mycelium samples were dried between tissue paper and frozen in liquid nitrogen. For RNA analysis only the 2 h samples were used. The supernatant was kept at -20°C for sugar and enzymatic analysis.

Sexual crossing of *A. nidulans*

Spores of the *A. nidulans* strains were harvested in saline tween buffer. Sexual crosses of the strains were performed as described previously [19]. Selection of the progeny was based on the inability to grow on D-glucose and D-fructose for the hexokinase glucokinase double mutant and a halo around a colony grown on starch plus D-glucose for the *creAΔ4* mutant. Selection of the triple mutant was based on the inability to grow on starch plus D-glucose and the ability to grow on starch plus D-xylose with the formation of a halo around the colony.

Enzyme and reducing sugar assays

The concentration of the sugars in the media was measured by a reducing sugar assay as previously described [20] with modifications: samples were mixed with an equal volume of 3,5-dinitrosalicylic acid (DNS), incubated at 95°C for 30 min and absorbance were measured at 540 nm using a microtiter plate reader (FLUOstar OPTIMA, BGM Labtech).

Extracellular enzyme activity was measured in a total volume of 100 µl using 0.01% *p*-nitrophenol (PNP) linked substrates, 20 µl of the culture samples and 50 µl of 50 mM sodium acetate pH 5.0. Samples were incubated in microtiter plates for 1 h at 37°C. Reactions were stopped by addition of 100 µl 0.25 M Na_2CO_3. Absorbance was measured at 405 nm in a microtiter plate reader (FLUOstar OPTIMA, BMG Labtech). The extracellular enzyme activity was calculated using a standard curve ranging from 0 to 40 nmol *p*-nitrophenol per assay per volume. Absorbance measurements for all assays were performed in triplicate. Statistical significance for all enzyme and reducing sugar assays was determined by t-test and the Holm-Sidak method (alpha = 0.05) using GraphPad Prism version 7 for Mac, GraphPad Software, www.graphpad.com.

Monosaccharide analysis

Monosaccharide analysis was performed for all strains, after 2, 8 and 24 h of cultures in wheat bran. The culture supernatant was diluted 10-fold in MilliQ water prior to analysis. The 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-equibration of 18 mM NaOH for 10 min (20°C; flow rate: 0.30 mL/min). 2.5-200 mM D-glucose, D-fructose, L-arabinose, D-xylose, D-mannose, L-rhamnose, D-galactose, D-glucuronic acid, and D-galacturonic acid (Sigma-Aldrich) were used as standards for quantification [21].

RNA extraction, cDNA library preparation and RNA-seq

Total RNA was extracted from mycelium ground in a Tissue Lyser (QIAGEN) using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. RNA integrity and quantity were analyzed on a 1% agarose gel using gel electrophoresis and with the RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA library preparation and sequencing reactions were performed by BGI Tech Solutions Co., Ltd. (Hong Kong). Illumina library preparation, clustering, and sequencing reagents were used throughout the process following the manufacturer's recommendations (http://illumina.com). On average 51 bp sequenced single-end reads were obtained, producing approximately 570 MB raw yields for each sample. Principal component analysis (PCA) was performed to show the reproducibility of our samples (Fig. S6).

RNA-seq data analysis and functional annotation

Raw reads were produced from the original image data by base calling. After data filtering, the adaptor sequences, highly 'N' containing reads (> 10% of unknown bases) and low quality reads (more than 50% bases with quality value of < 5%) were removed. After data filtering, in average, 95% clean reads remained in each sample. Clean reads were then mapped to the genome of *A. nidulans* (AspGD) using SOAPALIGNER/SOAP2 [22]. No more than two mismatches were allowed in the alignment. On average, 90% total mapped reads to the genome was achieved. The gene expression level was calculated by using RPKM method [23]. Genes with expression value higher than 150 were considered highly expressed (approximately top 5%) and differential expression was identified by CyberT bayesian ANOVA algorithm [24] with a cut-off value of fold change > 1.5 and P-value (corrected by multiple tests) < 0.05. The RNA-seq data have been submitted to Gene Expression Omnibus (GEO) [25] with accession number: GSE94775.

Orthologous genes between *Aspergillus niger* CBS 513.88 and *Aspergillus nidulans* FGSC A4 were obtained from AspGD [26] and FunCat [10] functional annotation was mapped accordingly. Expression higher than 150 RPKM that had a fold change of at least 1.5 between *creAΔ4 hxkA1 glkA4* vs *creAΔ4* and *hxkA1 glkA4* vs reference was analyzed regarding the functional annotation.

Quantitative RT-PCR (qRT-PCR) validation

RNA samples for RNA-seq experiments were used for qRT-PCR. cDNA was prepared from total RNA (2.5 µg) using Thermoscript RT (Invitrogen) according to the instructions of the manufacturer. The sequences of all primers for qRT-PCR analysis were designed using the Primer Express 3.0 software (Applied Biosystems). The primers were tested to determine the optimal primer concentrations and efficiency.

Combinations of the 50 nM, 300 nM and 900 nM (final concentration) per primer pair were checked and based on the dissociation curve the optimal primer concentration per primer pair was set. The primer sequences and optimal concentrations of the tested genes and the reference gene are listed in Table S5. qPCR analysis was performed by using the ABI 7500 fast real-time PCR system (Applied Biosystems). 20 µl reactions consisted of 2 µl forward and reverse primers at optimal concentration, 20 ng cDNA sample, 10 µl ABI Fast SYBR Master Mix (Applied Biosystems), and water to a final volume of 20 µl. The cycling parameters were 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. A dissociation curve was generated to verify that a single product was amplified. Transcript levels were normalized against the glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*; AN8041), expression and quantified according to the formula 2 –(Ct gene X – Ct gpd) [27]. Control reactions included water only and RNA (i.e. not converted to cDNA to detect residual DNA in the sample). Two biological and three technical replicates were analyzed.

Chemicals

All chemicals and reagents were acquired from Sigma-Aldrich unless otherwise noted.

Sample preparation for GC-MS metabolomics

The polar metabolites were extracted using 30 mg biomass from *A. nidulans* cell pellet, to which 200 µL MilliQ water and 800 µL of -20°C chloroform: methanol solution (2:1) was added. The cultures were vortexed for 10 seconds, sonicated for 10 min and then centrifuged (13,300 × g, 4°C, 5 min). After centrifugation 100 µL of the top aqueous layer was transferred to glass vials and dried in a vacuum concentrator (CentriVap Concentrator, Labconco). For the analysis of the polar metabolites in the spent media 100 µL of spent media was transferred directly to glass vial and dried in a vacuum concentrator.

Chemical derivatization and GC-MS analysis

Polar metabolites from both the cell pellet and spent media were derivatized as described previously [28]. Briefly, 20 µL of methoxyamine hydrochloride in pyridine (30 mg/mL) was added to each sample, followed by 30 seconds of vortexing and 10 seconds of sonicating before incubating the samples with shaking (1000 rpm) at 37°C for 90 min. Next, 80 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) was added to each sample, subsequently, the samples were vortexed for 30 sec, sonicated for 10 sec and incubation at 37°C with shaking (1000 rpm) for 30 min. Derivatized samples were then transferred to insert before being analyzed by GC-MS in random order. Blanks and FAMEs samples were also included in the analyses for background reference and RT calibration purposes, respectively.

The derivatized metabolites were separated using a HP-5MS column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Inc.) and analyzed on an Agilent GC 7890A coupled with a single quadrupole MSD 5975C (Agilent Technologies) system. For the GC-MS analysis, 1 µL of the samples was injected (splitless) with the GC oven kept at 60°C for 1 min after injection, subsequently, the temperature was increased to 325°C by 10°C/min, followed by a 5 min hold at 325°C. Throughout the analysis the injection port temperature was kept constant at 250°C for optimal analysis.

GC-MS data processing

The analysis of the polar metabolites was done by processing the GC-MS raw data files using Metabolite Detector as stated previously [29]. Briefly, the retention indices (RI) calibration was carried out based on the analyses of a mixture of FAMEs internal standards (C8-C28). The data from all GCMS runs were chromatographically aligned after deconvolution and referenced to the FAMEs analyses. In the first instance metabolites were identified by matching experimental spectra to a PNNL augmented version of FiehnLib (PMID: 19928838) library [30], containing spectra and validated retention indices of more than 900 metabolites. Furthermore, unidentified metabolites were screened against the NIST14 GC-MS Spectral Library by comparing spectra alone (denoted with “NIST”). Subsequently, all metabolite identifications were manually validated to reduce deconvolution errors during automated data-processing and to eliminate false identifications. The curated data set of identified metabolites, unidentified features and their abundances for each sample was then further analyzed making use of MetaboAnalyst [31] for the multivariate data analysis (MVDA). Data were median normalized and log transformed followed by principal component, hierarchical cluster, and heatmap analysis to identify natural clustering within the data.

Results

Construction and verification of the mutants

A hexokinase glucokinase double mutant (*hxkA1 glkA4*) and a triple mutant (*creAΔ4 hxkA1 glkA4*) in *A. nidulans* were obtained through sexual crosses (Table 1 and Fig. S1).
A reference strain with yellow conidial color (UUAM101.08) was selected from the progeny of the first cross, a *hxkA1 glkA4* mutant (UUAM102.37) was selected from the second cross, and a *creAΔ4* (UUAM104.47) as well as the triple mutant *creAΔ4 hxkA1 glkA4* mutant (UUAM104.87) were selected from the third cross for further analysis (as described in the Experimental procedures, Fig. S1).
Phenotypic analysis was performed to visualize the known growth defects and thus confirm the genetic mutations in our selected progeny strains.

All strains were grown on D-glucose, D-fructose and D-xylose, using appropriate supplements as indicated in the experimental procedures. As expected, growth of the glucokinase hexokinase double mutant and the triple mutant was impaired on D-glucose and D-fructose compared to the reference (Fig. 2A). The deletion of *creA* affected growth on all carbon sources tested (Fig. 2A-B). Moreover, its growth was reduced on starch and starch plus D-glucose compared to the reference, although a halo was observed around the colony which was absent in the reference (Fig. 2B). Halo formation on starch plates around the colony is indicative of the activity of starch-degrading enzymes, converting non-soluble starch into soluble oligosaccharides, effectively 'clearing' the media. In the presence of 3% D-glucose or D-xylose, CreA represses the production of these enzymes, resulting in the absence of a halo. However, when *creA* is deleted, the halo is observed even in the presence of 3% D-glucose or D-xylose. The triple mutant failed to grow on starch plus D-glucose but not on starch plus D-xylose where a halo was also observed around the colony (Fig. 2B). The double mutant was unable to grow on starch plus D-glucose like the triple mutant but grew well on starch and starch plus D-xylose. These results confirmed the expected phenotypes of the selected strains.

Growth profiling on plant biomass substrates revealed different phenotypes between the reference and the mutants

Growth of the reference strain and mutants was further analyzed on 12 pure polysaccharides and untreated agricultural waste substrates to determine the importance of glycolysis for growth (Fig. 3).

On all the selected carbon sources (except cottonseed pulp) the *creAΔ4* grew more strongly than the *hxkA1 glkA4* and *creAΔ4 hxkA1 glkA4* strains (Fig. 3). The hexokinase glucokinase negative mutant was unable to grow on guar gum and sugar beet pulp, whereas growth was strongly reduced on the other tested carbon sources.

On xylan, citrus pulp, guar gum, soybean hulls and in particular wheat bran and sugar beet pulp, growth of the carbon catabolite derepressed triple mutant was better than of the hexokinase glucokinase double mutant (Fig. 3). Thus, growth of the double mutant on these agricultural waste substrates can be partially restored by removing carbon catabolite repression. Wheat bran is an agricultural based crude substrate and a by-product of the wheat milling industry. It consists mainly of (glucurono)arabinoxylan, cellulose, and starch, so is a good source of fermentable sugars such as D-glucose, D-xylose, and L-arabinose. Because a strong difference in growth was observed between the double and triple mutant on wheat bran, this substrate was selected for further analysis in this study. The composition of the polymeric and crude substrates was analyzed (Table S1), but this did not reveal any obvious correlation between phenotype and the composition of the substrates.

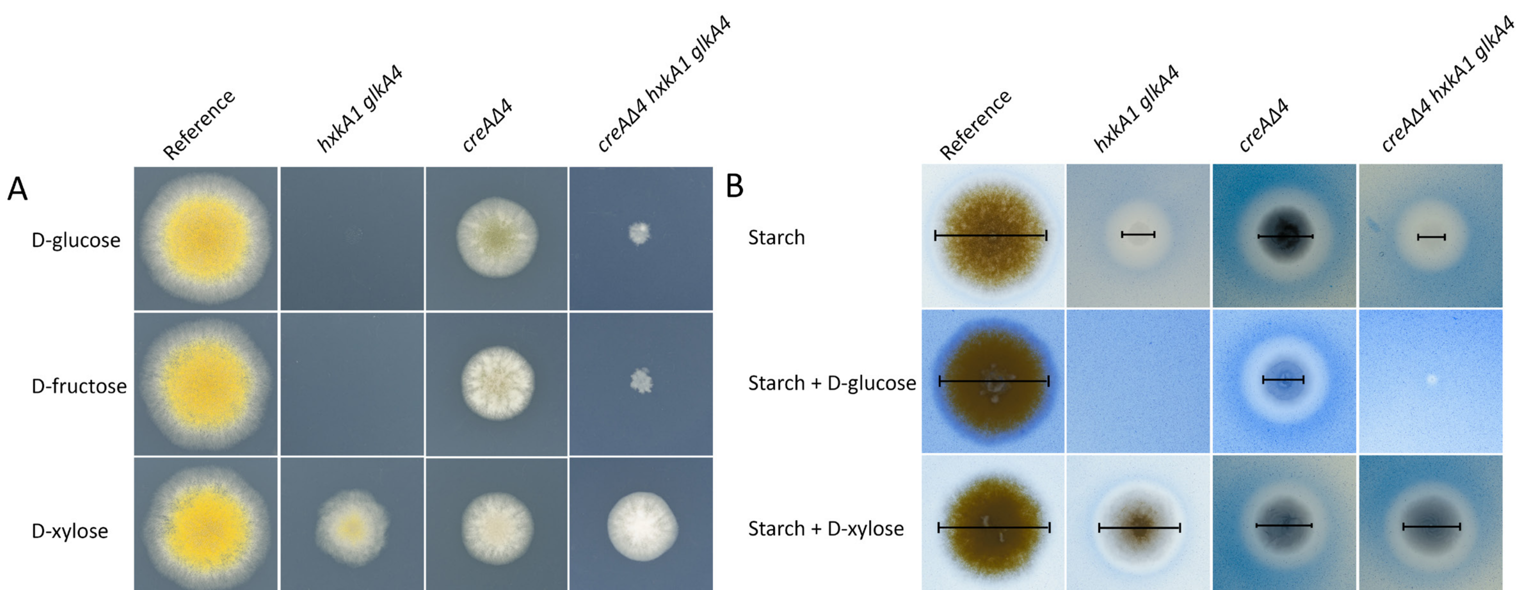

Figure 2 | Phenotype verification of *hxkA1 glkA4, creAΔ4 and creAΔ4 hxkA1 glkA4*
(A) Growth of the reference and the mutants on glucose, fructose and xylose. The *A. nidulans* strains were grown on MM with 25 mM D-glucose, 25 mM D-fructose and 25 mM D-xylose for 2.5 days at 37°C. (B) Growth of the reference and the mutants on starch. The *A. nidulans* strains were grown on MM with 1% starch with 1% starch azure, on 1% starch plus 3% D-glucose with 1% starch azure plus 3% D-glucose and on 1% starch plus 3% D-xylose) with 1% starch azure plus 3% D-xylose for 3 days at 37°C. Spore inoculations were done with $5x10^5$ spores/ml. The bars represent the diameter of the colonies.

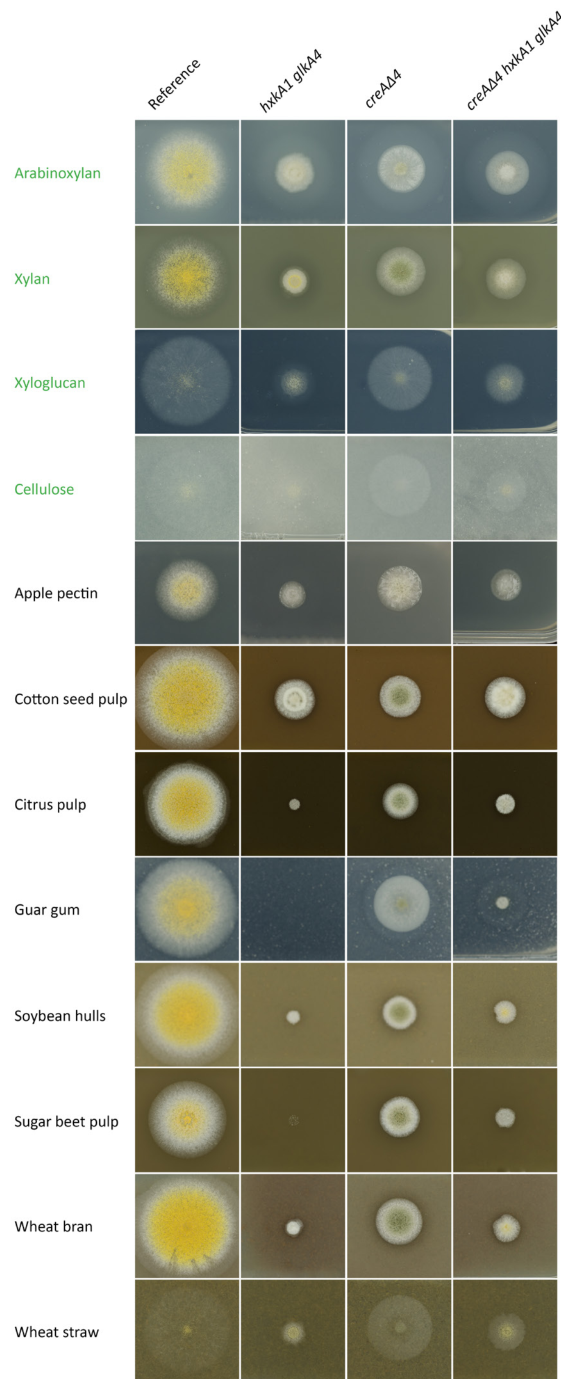

Figure 3 | Growth of the reference*, hxkA1 glkA4, creAΔ4 and creAΔ4 hxkA1 glkA4* strains on a selection of pure (in green) and crude substrates. Concentrations of the substrates were 1% for apple pectin, guar gum, xylan, arabinoxylan, cellulose, xyloglucan and 3% for cotton seed, citrus pulp, wheat straw, sugar beet pulp and wheat bran.

Sugars do not accumulate in cultures of *A. nidulans* reference and mutant strains grown on wheat bran

Accumulation of sugars in the extracellular medium after transfer of the reference and mutants on wheat bran was analyzed based on the number of reducing groups that could be detected. The results showed that a high extracellular sugar level was present for the triple mutant after 8 h of cultivation, whereas no sugars were detected for the reference and the *creAΔ4* strain itself. Sugars were also present in the extracellular medium of the double mutant after 8 h of transfer to wheat bran, but at five-fold lower concentration compared to the triple mutant (Fig. 4A). After 24 h of transfer to wheat bran, the sugar concentration in the triple mutant is reduced to a very low level (Fig. 4A). To determine any changes in sugar composition, all strains were pre-grown in liquid cultures containing MM and the necessary vitamins with D-xylose and then transferred to the same medium with wheat bran. The monosaccharide content in the media was analyzed by High-Performance Anion-Exchange Chromatography (HPAEC) with Pulse Amperometric Detection (PAD) (Fig. 4B-C-D).
Significant differences in glucose concentrations were found between the triple mutant and *creAΔ4* strain. After 8 h of transfer, a high level of glucose (31 mM) was detected in the triple mutant, while in the *creAΔ4* strain glucose was not detectable (Fig. 4B). The concentration of glucose was five-fold lower in the reference and double mutant compared to the triple mutant. The glucose levels in the reference and double mutant were similar at all the time points tested (2, 8 and 24 h) (Fig. 4B). In the *creAΔ4* strain, glucose was only detected after 2 h of transfer to wheat bran.
Xylose was not detected in cultures of the reference strain and the double mutant (Fig. 4C). After 2 h of transfer to wheat bran, a high level of xylose was detected in the *creAΔ4* (from 4 to 8 mM) and the triple mutant (6,5 mM), which was reduced in both mutants after 8 h (Fig. 4C). This trend was also observed in both *creAΔ4* and triple mutant for arabinose, but at a very low level (Fig. 4D). Low levels of arabinose were also detected in the reference and double mutant at all the time points tested (Fig. 4D). After 24 h of transfer to wheat bran no glucose, xylose or arabinose was detected in the media of all the strains tested (Fig. 4B-C-D). Thus, our analysis revealed that sugars are not progressively accumulating extracellularly but are consumed during growth of both the double and triple mutants on wheat bran. This is expected for D-xylose and L-arabinose but the absence of free glucose after 24 h suggests that alternative pathways are converting the glucose.

To trace the conversion products of glucose, GC-MS metabolomic analysis was performed on intracellular metabolite samples from the mycelia of *A. nidulans* at 2, 8 and 24 h after transfer to the 1% wheat bran media. 134 metabolites were detected (Table S2), of which 79 could be identified by matching them to entries in the Agilent Fiehn Metabolomics RTL and NIST GC-MS libraries. Metabolite analysis in the *creAΔ4* and triple mutants showed intracellular accumulation of glucose after 2 h. After 8 h the level is lower and after 24 h no glucose was detected (Fig. S2).

For fructose, we also observed an intracellular accumulation in the *creAΔ4* and triple mutant after 2 h of transfer to wheat bran. After 8 h, the accumulation was only observed in the *creAΔ4* mutant, and at 24 h no fructose was detected. Identified metabolite profiles related to sugar catabolism in *A. nidulans* were similar for the *hxkA1 glkA4* mutant compared to the reference and for *creAΔ4* compared to the triple mutant. After 24 h of transfer to wheat bran medium, no glucose or fructose was detected in any of the strains (Table S2). In summary, the hexokinase glucokinase deficient strains when grown on a complex substrate like wheat bran did not show any effect at the metabolite levels after 24 h of growth that indicated accumulation of glycolytic intermediates.

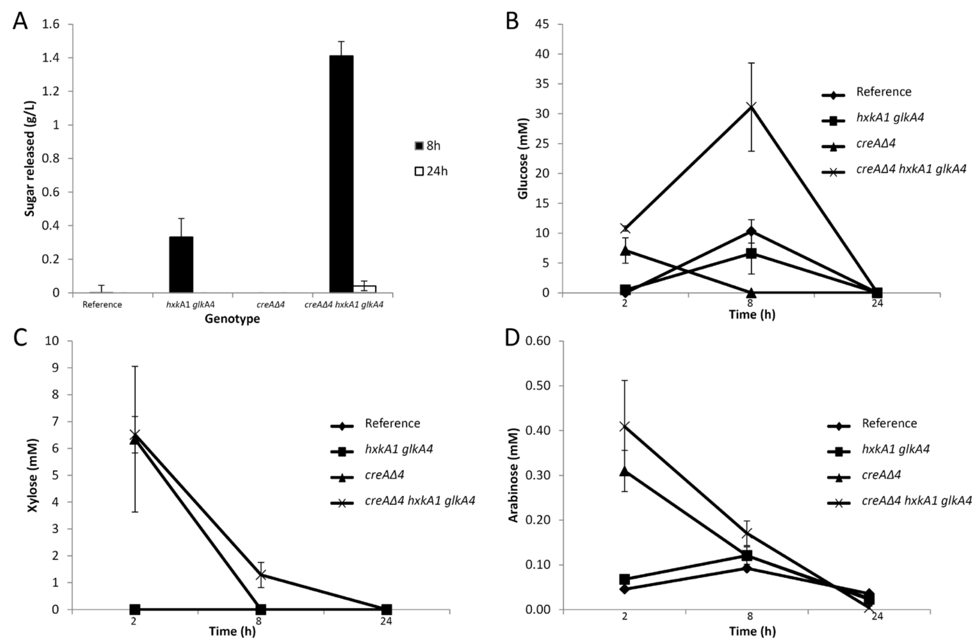

Figure 4 | Sugar consumption by *Aspergillus nidulans* reference and mutants.
All *A. nidulans* strains were transferred to 1% wheat bran.
(A) Sugar levels in the extracellular medium of the reference, *hxkA1 glkA4, creAΔ4 and creAΔ4 hxkA1 glkA4* after 8 h and 24 h of transfer to wheat bran.

(B) HPAEC-PAD monosaccharide analysis of glucose, (C) xylose and (D) arabinose from *A. nidulans* reference (dark circle), *hxkA1 glkA4* (dark square), *creAΔ4* (dark triangle) and *creAΔ4 hxkA1 glkA4* (dark cross) after 2, 8 and 24h of transfer to wheat bran. A significant difference was identified between the triple mutant and *creAΔ4* for glucose after 8 h ($p>0.05$ in students t-test). Means and SD (errors bars) were calculated from three technical replicates.

Most of the up-regulated genes in the triple mutant compared to the *creAΔ4* strain are assigned to the Metabolism class

RNA-sequencing was carried out using RNA isolated from D-xylose pre-grown mycelium from all four strains which were then transferred to MM with the necessary supplements and 1% wheat bran to examine changes in gene expression profiles as a result of the hexokinase glucokinase defect in two genetic backgrounds (reference and *creAΔ4*). Thus in the next paragraphs, we compare the 3 mutants to the reference strain, and the same for the *creAΔ4* background.

To classify the function of the predicted *A. nidulans* genes, the functional catalogue (FunCat) was used [10]. Out of the total 10460 genes predicted in the genome of *A. nidulans*, 5812 were associated with FunCat (component, function, and process) that were assigned to 19 main groups (see legend Fig. S3). There were 1798 genes up-regulated genes in the triple mutant compared to the *creAΔ4* strain, while 814 were up-regulated in the double mutant compared to the reference. Considering all up-regulated genes together, Metabolism was the major class with 39%, followed by Unclassified Proteins (34%), Transcription (5.5%) and Cellular Transport and Transport Mechanisms (5%).

Analysis of the genes from the Metabolism class was made in these two comparisons. There were 8 subgroups found in this FunCat class (see legend Fig. S3). C-compound and carbohydrate metabolism was found to be the largest subgroup in the Metabolism class with 43% (307 genes) in the triple mutant vs the *creAΔ4* strain of the total of up-regulated genes (Fig. S3). In the double mutant, 50% of the up-regulated genes in the Metabolism class belong to the C-compound and carbohydrate metabolism subgroup (170 genes). Gene function of all genes assigned to the C-compound and carbohydrate metabolism subgroup was analyzed (Table S3). Among the up-regulated genes assigned to the C-CM group, the *swoM* gene encoding the glucose-6-phosphate isomerase, two genes involved in the PCP (xylitol dehydrogenase: *xdhA* and L-arabitol dehydrogenase: *ladA*) and four genes involved in the PPP (D-ribulokinase: *rbtA*; D-ribulose-phosphate-3 epimerase: *rpeA*; glucose-6-phosphate-1-dehydrogenase: *gsdA* and transaldolase: *pppA*) were up-regulated in the triple mutant compared to *creAΔ4* (Table S3, sheet 1). Whereas, in the double mutant (Table S3, sheet 2), an additional gene involved in the PPP (6-phosphogluconate dehydrogenase: *gndA*) was up-regulated compared to the reference.

The reduced ability to grow on wheat bran is not due to a reduced expression of CAZy genes involved in plant biomass degradation

The CAZy genes involved in plant biomass from the Glycoside Hydrolase (GH), Auxilary Activities (AAs), Carbohydrate Esterases (CEs) and Polysaccharide Lyases (PLs) families represented 79%, 7%, 4% and 10%, respectively, of all the CAZymes encoding genes. Those genes represent 1.7% of the total number of genes identified in our RNA-sequencing.

CAZy genes with RPKM values above 150 in any of the four conditions are represented in red in Table S4 (sheet 2). Most of these highly expressed CAZy genes are involved in xylan and cellulose degradation. Among the significantly differentially expressed genes (with >1.5 fold change and p-value <0.05), there are only 17 down-regulated and 10 up-regulated genes in the *hxkA1 glkA4* mutant compared to the reference after a transfer to wheat bran for 2h (Table S4, sheet 1). Interestingly, 6 out of 17 genes involved in starch degradation were down-regulated in the double mutant and these genes include four α-glucosidases (*agdA*, *agdB*, *agdE* and *agdG*), one α-amylase (*amyA*) and one glucoamylase (*glaA*). In addition, two endo-xylanases (*xlnA* and *xlnC*), two endo-polygalacturonases (*pgaB* and *pgxA*) and one endo-arabinanase (*abnC*) were down-regulated. Three genes, two α-amylase genes (*amyD* and *amyG*) and one α-glucosidase gene (*agdF*) involved in starch degradation are up-regulated in the *hxkA1 glkA4* mutant as well as seven other CAZy genes that encode all exo-acting enzymes involved in degradation of xylan, cellulose/xyloglucan, galactomannan and pectin.

Overall the reference and the double mutant showed the same CAZy expression profile, but these differed from the two *creAΔ4* background strains. As expected, many CAZy genes are up-regulated at high transcript levels in the *creAΔ4* and the triple mutant (Table S4, sheet 1). Between these two strains there are 16 genes down-regulated and 33 genes up-regulated. Most of the down-regulated genes are involved in starch (8 genes) and cellulose/ xyloglucan (7 genes) degradation. We would like to note that even though expression of these genes was significantly reduced, transcript levels of many genes remain at a high level in the triple mutant. Transcript levels of 7 out of 16 genes involved in arabinoxylan degradation were even more increased in the triple mutant, including the endo-xylanase gene *xlnA*, four putative β-xylosidases (AN1870, AN7864; *bxlD*, AN8477 and AN2664) and two α-arabinofuranosidases (*abfB* and AN7781). In summary, these results indicate that *creAΔ4* mutant has a stronger effect on CAZy gene expression than the metabolic mutations.

A dysfunctional *creAΔ4* mutant combined with blocking glycolysis results in an increased expression of pentose catabolic and pentose phosphate pathway genes (PCP and PPP)

Most glycolytic genes showed a similar profile with a significant difference in expression between the reference and the double mutant and between *creAΔ4* and the triple mutant. As expected, in both mutants in which glycolysis is blocked the expression of 6-phosphofructokinase (*pfkA*), fructose-biphosphate aldolase (*fbaA*), glyceraldeyde-3-phosphate dehydrogenase (*gpdA*), phosphoglycerate kinase (*pgkA*), phosphoglycerate mutase (*pgmA*) and enolase (*acuN*) were down-regulated (lower than 0.7-fold). Exceptions were glucose-6-phosphate isomerase (*swoM*) and pyruvate kinase (*pkiA*), which were higher expressed in the hexokinase glucokinase double mutant in both the reference and the *creAΔ4* background (Fig. 5 and Table S5).

Two genes from the pentose catabolic pathway (PCP), *xdhA* and *ladA*, were up-regulated in the double mutant compared to the reference and in the triple mutant compared to *creAΔ4* (> 1.5 fold, p-value <0.05) (Table S5). In addition, five PPP genes had a significantly higher expression in the double mutant compared to the reference and in the triple mutant compared to *creAΔ4.* These encode transaldolase (*pppA*), D-ribulokinase (*rbtA*), D-ribulose-phosphate-3 epimerase (*rpeA*), 6-phosphogluconate dehydrogenase (*gndA*) and glucose-6-phosphate-1-dehydrogenase (*gsdA*). Wheat bran contains also other monosaccharides such as galactose, mannose and glucuronic acid. However, genes involved in the Leloir pathway, alternative D-galactose pathway, D- mannose pathway and TCA cycle did not show a clear effect of blocking glycolysis at the level of hexose phosphorylation.

No difference in gene expression of other genes that might convert glucose, such as glucose oxidase, glucose dehydrogenase, gluconate dehydratase and several hexokinases putative (HxkB, HxkC, HxkD), was observed in the double and triple mutant compared to their reference strains (Table S5). In our metabolomics data, we could not find any other intermediates pointing to an alternative pathway for the conversion of glucose.

Validation of the RNA-seq expression profiles by quantitative RT-PCR (qRT-PCR)

To validate the expression profiles obtained by RNA-seq analysis, two genes involved in the PCP (xylitol dehydrogenase, L-arabitol dehydrogenase) and one CAZy gene (α-arabinofuranosidase; *abfB*) were tested by quantitative RT-PCR (Fig. S4). Expression trends of the selected genes obtained by qRT-PCR confirmed those obtained with RNA-seq. The primers used in this experiment are indicated in Table S6.

6

Higher α-arabinofuranosidase and β-xylosidase activities were observed in the triple mutant compared to the *creAΔ4* mutant alone

The same cultures used for RNA-sequencing were also used to measure α-arabinofuranosidase, β-glucosidase, β-xylosidase and cellobiohydrolase activities in the supernatants of the cultures 8 and 24 h after transfer to wheat bran medium. After 8 h of transfer, cellobiohydrolase (CBH) and β-glucosidase (BGL) activities were significantly higher in the reference and the *creAΔ4* compared to the two other mutant strains, respectively (Fig. S5). After 24 h of transfer to wheat bran, this difference was not observed anymore and cellulolytic activity levels were similar in all strains.

A significant increase in β-xylosidase (BXL) and α-arabinofuranosidase (ABF) activities was observed in the triple mutant in comparison with the *creAΔ4* after 8 and 24 h (Fig. S5). No differences in BXL activity were observed between the reference and the double mutant at both time-points.

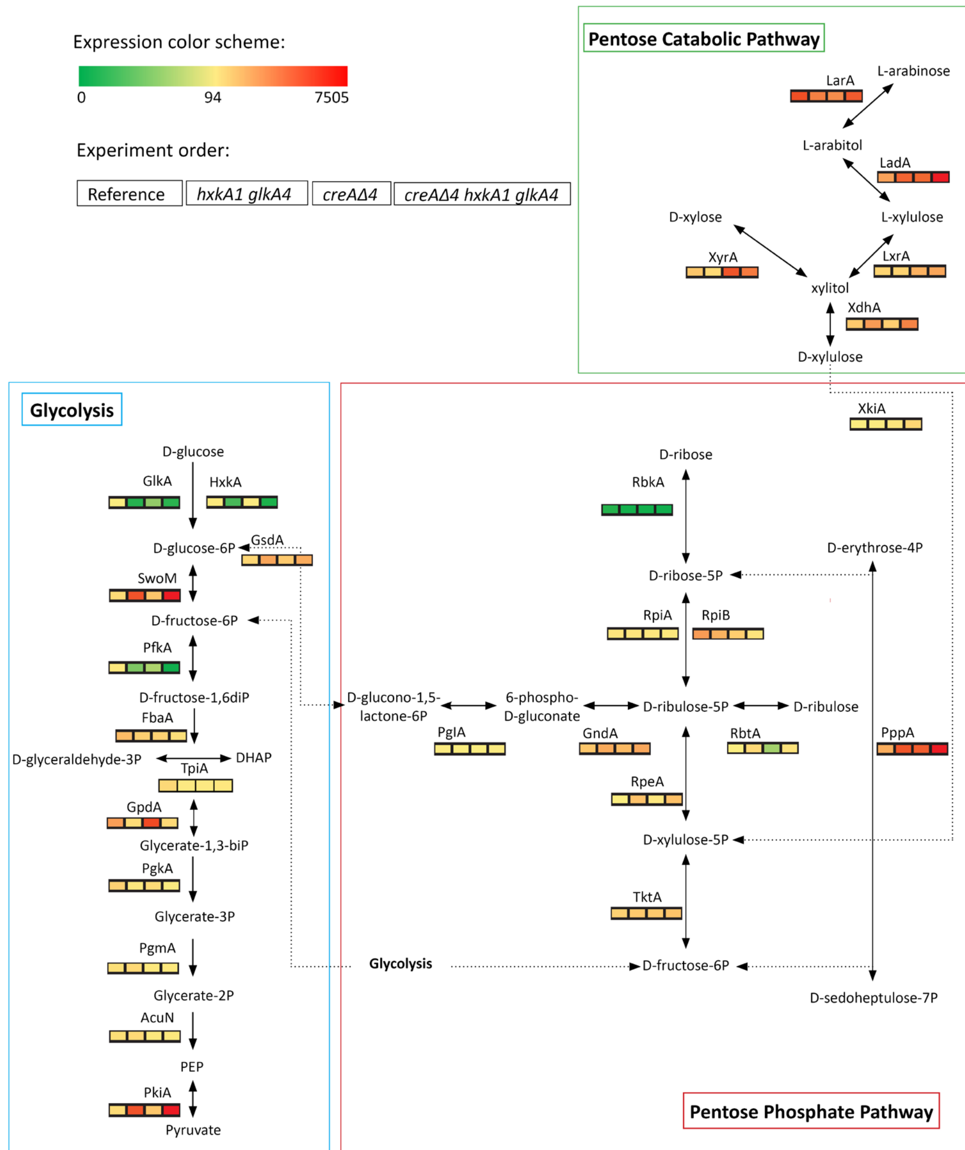

Figure 5 | Schematic representation of the expression of genes of glycolysis, pentose phosphate pathway (PPP) and pentose catabolic pathway (PCP) in *A. nidulans* after 2 h of transfer to 1% (w/v) wheat bran. Gene expression values are presented under the genes and indicated by a color gradient. A decrease in expression is indicated by green squares and increased expression is indicated by a red square. Gene expressions are average values of two biological replicates. The units used is fragments per kilobase of exon per million reads mapped (FPKM). Genes involved in the glycolysis: glucokinase (*glkA*; AN8689), hexokinase (*hxkA*; AN7459), glucose-6-phosphate isomerase (*swoM*; AN6037), phosphofructokinase (*pfkA*; AN3223), fructose-biphosphate aldolase (*fbaA*; AN2875), dihydroxyacetone phosphate (DHAP), triose-phosphate isomerase (*tpiA*; AN6900), glyceraldeyde-3-phosphate dehydrogenase (*gpdA*; AN8041), phosphoglycerate kinase (*pgkA*; AN1246), phosphoglycerate mutase (*pgmA*; AN3059), enolase (*acuN*; AN5746) and pyruvate kinase (*pkiA*; AN5210). Genes involved in the PCP: L- arabinose reductase (*larA*; AN7193), L-arabitol dehydrogenase (*ladA*; AN0942), L-xylo-3-hexulose reductase (*lxrA*; AN10169), D-xylose reductase (*xyrA*; AN0423), xylitol dehydrogenase (*xdhA*; AN9064) and D-xylulose kinase (*xkiA*; AN8790).

Genes involved in the PPP: glucose-6-phosphate-1-dehydrogenase (*gsdA*; AN2981), 6-phosphogluconolactonase (*pglA*; AN0285), 6-phosphogluconate dehydrogenase (*gndA*; AN3954), D-ribulose-phosphate-3 epimerase (*rpeA*; AN7588), transketolase (*tktA*; AN0688), D-ribulokinase (*rbtA*; AN6985), transaldolase (*pppA*; AN0240), ribose 5-phosphate isomerase (*rpiA*; AN2440), ribose 5-phosphate isomerase (*rpiB*; AN5907) and ribokinase (*rbkA*; AN7995).

Discussion

In this study, our aim was to create an *Aspergillus* strain that more rapidly releases hexoses from plant biomass but does not metabolize them through glycolysis, so they can be fermented by yeast more efficiently to produce bio-fuels. As a first step to achieve this, mutants for hexokinase, glucokinase, *creA* and combinations thereof were constructed in *A. nidulans* and the resulting strains were evaluated for their ability to accumulate hexoses. HxkA and GlkA represent the only active hexose kinases, even though other genes encoding putative hexose phosphorylating activities, such as the putative HxkB, are present in the genome of *A. nidulans*. A previous study in *Aspergillus fumigatus* reported that recombinant HxkB has no sugar-phosphorylating activity [8].
The phenotype of the *hxkA1 glkA4* strains was confirmed [5] and the double mutant was shown to be unable to grow on D-glucose and D-fructose. The *creAΔ4* has been described as a strongly derepressed *cre*A mutant [11] and was shown to have elevated polysaccharide degrading enzyme activities [12]. Our RNA-seq data indicate that this is a transcriptionally down-regulated *cre*A allele.
First, we evaluated the changes in the extracellular sugars in the reference and in all the mutant strains. A DNS sugar assay showed that free sugars were only present in the culture supernatant at the early time point in the triple mutant, while after 24 h of incubation, sugar levels were close to zero in all the strains. This suggests that the balance of sugar release and sugar uptake had reached a stable state. This is in agreement with the results obtained from the HPAEC analysis. At 8 h, glucose was higher in the triple mutant compared to the *creAΔ4*, but after 24 h no glucose was detected in any of the strains. Interestingly, fructose and other monosaccharides did not accumulate in all strains after 2, 8 and 24 h of transfer to wheat bran. This indicates that hexoses are still being consumed in the *hxkA1 glkA4* negative strains suggesting that alternative pathways are converting them.

Next, we studied differentially CAZy gene expression after a 2h transfer of the reference and mutant strains to wheat bran. As expected, the results showed that the *creAΔ4* mutant has a strong effect, i.e. transcript levels were highly induced, on CAZy gene expression because carbon catabolite repression via CreA is affected. Interestingly, the *hxkA1 glkA4* mutation showed to have an effect on CAZy gene expression profiles and it correlates to the changes in extracellular enzyme activity profiles and transcript levels observed at the metabolic level.

Transcript levels of genes encoding arabinoxylan degrading enzymes (β-xylosidases; BXLs and α-arabinofuranosidases; ABFs) were increased in the triple mutant after 2h of transfer to wheat bran. After 8 and 24h, BXL and ABF activities remain at a higher level in the triple mutant compared to the *creAΔ4* strain. Two genes from the PCP, *xdhA* and *ladA*, were up-regulated in both *hxkA1 glkA4* negative strains suggesting an increasing flux through this pathway.

In the *hxkA1 glkA4* mutant, a set of genes encoding starch degrading enzymes was down-regulated at 2h on wheat bran compared to the reference. In the triple mutant, expression of both starch and cellulose genes were reduced compared to the *creAΔ4* strain. Most glycolytic genes were down-regulated in both double and triple mutant as well suggesting blocking glycolysis causes an initial negative feedback of extracellular D-glucose release from starch and cellulose. The β-glucosidase (BGL) and cellobiohydrolase (CBH) activities also decreased after 8h of transfer to wheat bran in both *hxkA1 glkA4* negative strains. After 24h of incubation, BGL and CBH enzyme activities were at a similar level as their reference strain. Together with the extracellular sugar analysis, this again suggests that during growth on wheat bran in time, a reorganization of central metabolism occurs in the *hxkA1 glkA4* negative strains to regulate D-glucose release and re-direct glucose conversion.

Growth profiles of the *hxk1 glkA4* negative strains on plant biomass and pure polysaccharides were performed to determine the importance of glycolysis for growth on these substrates. Previously it was shown that the *Aspergillus fumigatus* hexokinase glucokinase double deletion mutant failed to grow on glucose, fructose, mannose, sorbose, glucosamine, and saccharose [8]. Our results showed that in all the carbon sources tested (except arabinoxylan, xyloglucan, and cotton seed pulp) the double mutant had reduced growth compared to the triple mutant. The improved growth of our triple mutant in comparison with the double mutant observed for most substrates is most likely a consequence of the increased transcript levels of CAZymes in the *creAΔ4* mutant.

Our extracellular sugar analysis suggests that D-glucose and D-fructose may be converted through an alternative metabolic pathway. RNA-sequencing analysis allowed us to analyze in more detail the effect of the metabolic mutations and to possibly identify the pathway through which D-glucose and D-fructose were converted. Since in this biomass the amounts of galactose and mannose are low, these pathways will not substantially contribute to growth. Therefore, the PCP and PPP are the most relevant pathways necessary for growth. We assumed considering the metabolic defects introduced that sugar transport is not impaired in the set of strains used.

Most of the glycolytic genes were down-regulated in the *hxkA1 glkA4* strains in both the reference and *creAΔ4* background. However, glucose-6-phosphate isomerase, encoded by *swoM*, which converts glucose-6-phosphate to fructose-6-phosphate in a reversible reaction, was significantly up-regulated in the strains not able to phosphorylate D-glucose.
The PPP generates besides NADPH both fructose-6-phosphate and glyceraldehyde-3-phosphate.

The strong up-regulation of *swoM* indicates the necessity to increase the flux through the PPP by generating glucose-6-phosphate from the available fructose-6- phosphate pool. This is also indicated by the up-regulation of the expression of two out of three steps of the oxidative PPP encoded by *gsdA* and *gndA* which is most outspoken in the strains with a hexokinase glucokinase negative background. The pentoses D-xylose and L-arabinose are the main carbon sources available for catabolism and these pathways require NADPH. Xylose and arabinose represent 34% and 16% (w/w), respectively of the wheat bran. Xylitol dehydrogenase, encoded by *xdhA*, which converts xylitol into D-xylulose and L-arabitol dehydrogenase, encoded by *ladA*, which converts L-arabitol into L-xylulose were significantly up-regulated in the *hxkA1 glkA4* negative strains. Also, D-xylulose kinase, encoded by *xkiA*, which converts D-xylulose into D-xylulose-5-phosphate, was higher in the triple mutant. An important response in the non-oxidative PPP is also observed in the *hxkA1 glkA4* negative backgrounds which showed an increased expression of *rpeA* encoding the epimerase responsible for converting D- xylulose-5-phosphate into D- ribulose-5-phosphate and the *pppA* gene encoding a transaldolase. The ribose-5-phosphate isomerase encoding gene *rpiA* hardly changes its response in the mutant backgrounds whereas *rpiB* expression is the highest in the reference strain. Transcriptional regulation seems not to be necessary at this level. Subsequently, in the final steps of the pathway fructose-6-phosphate and glyceraldehyde-3-phosphate are generated. Glyceraldehyde-3-phosphate is then available for the final part of the glycolytic pathway which can then enter the TCA cycle.

The pool of fructose-6-phosphate generated by the catabolism of the pentoses will, in accordance with the up-regulation observed in the oxidative part of the PPP, be partially recycled whereas it will also be used for biosynthetic purposes by converting fructose-6-phosphate to glucose-1-phosphate via a glucose-6-phosphate isomerase (SwoM) and a phosphoglucomutase (PgmB). The *pgmB* gene and subsequent steps are not up-regulated though. However, a putative UTP-glucose-1-phosphate uridylyltransferase, encoded by *galF*, which converts glucose-1P into UDP-glucose, was up-regulated in the triple mutant. Also, a putative glycogen synthase, which converts UDP-glucose into glycogen, was up-regulated in the double and triple mutant. An excess of the fructose-6-phosphate pool may lead to fructose-6-phosphate phosphatase activity. Interestingly, a fructose-2,6-bisphosphate 2-phosphatase (*fbpZ*) was up-regulated in the triple mutant compared to the *creAΔ4* strain. This is likely to lead to lower levels of fructose-2,6-biphosphate (F2,6BP) and as a consequence to a diminished activation of the fructose-6-phosphate kinase [13]. Fructose observed intracellularly at early time points in the *creAΔ4* strain and the triple mutant may arise by fructose-6-phosphate phosphatase activity which could be the consequence of an accumulation of the fructose-6-phosphate pool. However, the presence of a glucose isomerase that could convert D-glucose into D-fructose cannot be excluded.

These results could explain the strong up-regulation observed in the *hxkA1 glkA4* strains in both the reference and *creAΔ4* background of sorbitol dehydrogenase, encoded by *sdhA*, which converts D-fructose into sorbitol. Therefore, the conversion of fructose-6-phosphate may occur via a phosphatase and subsequently SdhA. Another alternative for the fructose-6-phosphate conversion might be through mannitol-1-phosphate 5-dehydrogenase, encoded by *mdpA*, which converts fructose-6-phosphate into mannitol-1-phosphate. The *mdpA* gene is highly expressed in all strains. The accumulation of mannitol in *A. nidulans* when cultivated on glucose or fructose due to mannitol-1-phosphatase activity is well known [14-17]. Extracellular metabolite analysis showed high levels of mannitol in the *creAΔ4* and in the triple mutant after 2h of transfer to wheat bran (data not shown).

In summary our hypothesis is that D-xylose and L-arabinose are converted into fructose-6-phosphate and glyceraldehyde-3-phosphate. Fructose-6-phosphate will be converted into glucose-6-phosphate through the PPP, into mannitol through MpdA and mannitol-1-phosphatase as well as sorbitol through a phosphatase and subsequently SdhA. Expression data suggests the latter pathway to prevail. The RNA-seq results were also used to evaluate the expression of other metabolic genes that might convert D-glucose. Glucose oxidase activity would result in the formation of D-gluconate and hydrogen peroxide. Our RNA-seq data did not show a significant up-regulation in the double mutant compared to the reference strain and in the triple mutant compared to the *creAΔ4*. Also, in the metabolomics analysis we did not detect gluconate. Since D-glucose seems not to be oxidized, another option would then be reduction to glucitol. In the metabolomic analysis we also did not detect glucitol. D-glucose could also be converted into trehalose, since we observed formation of glucose-6-phosphate from the PPP whereas also glucose-1-phosphate and UDP-glucose biosynthesis is intact. But most likely, D-glucose is either directly reduced or converted to fructose followed by production of polyols.
In addition, our RNA-seq results showed a lack of response in the Leloir pathway, probably due to the low amount of galactose present in wheat bran.

In conclusion, this study provides an in-depth analysis of the effect of a hexokinase glucokinase double mutant at the gene expression level during growth of *A. nidulans* on an agricultural waste product. Although D-glucose and D-fructose initially accumulate, these hexoses are converted through alternative metabolic pathways. A more detailed metabolomic analysis is required to determine which pathways are actually used and what the final products are. *creAΔ4* mutant in combination with a hexokinase glucokinase deficiency results in an increased expression of the PCP and PPP. This indicates that the reduced ability to use hexoses as carbon source has resulted in a shift towards the pentose fraction of wheat bran as the major carbon source to support growth.

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Supplementary material

Table S1 | Composition of the crude substrates used in this study.
α-cellulose (C); Apple pectin (AP); Cotton seed pulp (CSP); Citrus pulp (CP); Guar gum (GG); Soybean hulls (SBH); Sugar beet pulp (SBP); Wheat bran (WB); Wheat straw (WS)

Mol %	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Total
C	0	2.50	47.40	10.60	0.80	38.70	0	
AP	0.86	1.58	0.96	0	4.84	4.38	86.36	97.42
CSP	0.60	2.19	49.97	0	1.17	39.28	6.79	49.19
CP	2	10.91	3.84	4.08	6.59	39	34.86	55.67
GG	0	2.22	0.40	56.91	35.44	3.19	1.67	83.61
SBH	1.00	8.44	15.03	7.06	3.95	50	15.91	68
SBP	1.46	29	2.37	2.12	6.53	32	27	56
WB	0	16.51	34.64	1.42	1.68	42.48	3.27	53.73
WS	0	4.97	37.02	1.02	1.59	51.06	4.34	57.10

Table S2 | Intracellular metabolism of *A. nidulans* during growth in wheat bran. **Available upon request from the author.**

Table S3 | Functional classification of *A. nidulans* genes belonging to the C-compound and carbohydrate metabolism subclass according to FunCat. **Available upon request from the author.**

Table S4 | Expression of selected CAZymes involved in the degradation of plant biomass in *A. nidulans.* **Available upon request from the author.**

Table S5 | Expression of known gene involved in central carbon metabolism in *A. nidulans.* (**See below)**

Table S6 | Primers used in this study to generate the gene fragments for qRT-PCR analysis. **Available upon request from the author.**

Figure S1 | Schema of sexual crossing in *A. nidulans.* (**See below)**

Figure S2 | Metabolomics quantification of selected metabolites from the glycolysis pathway**.** (**See below)**

Figure S3 | Functional classification of *A. nidulans* genes according to FunCat. **(See below)**

Figure S4 | Expression patterns and validation of RNA-sequencing analysis by qPCR. **Available upon request from the author.**

Figure S5 | Comparison of extracellular enzyme activities in reference and disruption strains. (**See below)**

Figure S6 | Principal component analysis. **Available upon request from the author.**

Table S5 | Expression of known gene involved in central carbon metabolism in *A. nidulans*. The expression higher than 150 is considered highly expressed and marked dark grey. The genes with expression lower than 20 are considered low expressed and marked light grey. The cut-off for differential expression is fold change >1.5 (cells marked dark grey = up-regulated) and fold change <0.7 (cells marked light grey= down-regulated) and p-value <0.05(*).

Pathway	Abbrv.	Gene number	Reference	*hxkA1 glkA4*	*creAΔ4*	*creAΔ4 hxkA1 glkA4*	*creAΔ4 hxkA1 glkA4/creAΔ4*	*hxkA1 glkA4*/Ref
Glycolysis	*glkA*	AN8689	104.38	29.27	62.74	31.88	0.51*	0.28*
	hxkA	AN7459	191.07	41.54	148.19	25.98	0.18*	0.22*
	swoM	AN6037	474.25	3648.45	976.32	5828.69	5.97*	7.69*
	pfkA	AN3223	209.88	56.53	70.86	18.87	0.27*	0.27*
	fbaA	AN2875	1199.06	768.67	701.11	390.90	0.56*	0.64*
	tpiA	AN6900	592.25	301.41	279.82	169.44	0.61*	0.51*
	gpdA	AN8041	1895.57	497.60	3854.07	541.68	0.14*	0.26*
	pgkA	AN1246	698.27	217.21	497.85	147.58	0.30*	0.31*
	pgmA	AN3059	99.85	68.75	148.54	78.19	0.53*	0.69
	acuN	AN5746	695.73	212.24	734.85	116.34	0.16*	0.31*
	pkiA	AN5210	200.15	249.98	339.93	244.17	0.72*	1.25
PCP	*xyrA*	AN0423	533.54	366.23	1530.28	1193.02	0.78*	0.69
	xdhA	AN9064	515.63	898.10	458.53	1119.64	2.44*	1.74*
	xkiA	AN8790	198.65	219.46	267.66	384.92	1.44*	1.10
	ladA	AN0942	851.25	1386.71	1351.14	2363.45	1.75*	1.63*
	lxrA	AN10169	412.35	435.21	713.66	799.30	1.12	1.06
	larA	AN7193	1589.13	1143.70	1023.66	1469.72	1.44*	0.72*
PPP	*gsdA*	AN2981	732.35	2213.50	1151.37	2029.25	1.76*	3.02*

	pglA	AN0285	171.56	143.11	134.02	170.59	1.27*	0.83
	gndA	AN3954	1242.58	2057.24	1622.58	2113.15	1.30*	1.66
	rpeA	AN7588	117.01	1314.35	284.12	1408.56	4.96*	11.23*
	rbkA	AN7995	14.14	5.22	2.78	2.40	0.86	0.37*
	rpiA	AN2440	367.44	360.01	302.60	276.84	0.91	0.98
	rpiB	AN5907	2476.50	1806.95	1004.28	374.65	0.37*	0.73
	tktA	AN0688	870.89	1165.96	1161.37	1350.96	1.16	1.34
	rbtA	AN6985	97.84	649.32	61.87	415.63	6.72*	6.64*
	pppA	AN0240	1958.26	4657.83	4367.05	7505.39	1.72*	2.38*
Leloir pathway		AN3184	397.56	475.01	780.95	747.00	0.96	1.19
		AN3432	228.77	153.07	484.13	457.07	0.94	0.67*
	galE	AN4957	110.12	83.09	54.72	55.11	1.01	0.75
	galD	AN6182	70.12	42.81	48.37	37.55	0.78*	0.61*
	galF	AN9148	297.03	587.33	347.81	483.95	1.39*	1.98*
	ugmA	AN3112	150.46	266.32	132.80	188.49	1.42	1.77*
	pgmB	AN2867	400.91	509.29	201.86	202.89	1.01	1.27
		AN4591	131.78	109.60	45.05	54.43	1.21*	0.83
	galGc	AN3119	45.46	153.86	5.38	3.71	0.69*	3.38*
Alternative D-galactose pathway	*ladB*	AN4336	24.09	29.66	0.33	1.16	3.56*	1.23
	xhrA	AN6450	2.54	4.96	0.63	2.24	3.54*	1.95*
	srdA	AN2666	13.54	3810.13	35.97	3481.95	96.81*	281.46*

Other genes								
		AN7890	11.37	13.87	12.11	15.84	1.31*	1.22
		AN4006	48.58	38.68	28.24	25.30	0.90	0.68
	gudB	AN4186	41.20	30.68	4.24	5.44	1.28	0.74
		AN6035	55.87	39.06	51.64	46.44	0.55*	0.70
		AN8010	277.17	533.64	124.59	332.95	2.67*	1.93*
	fbpZ	AN10844	154.78	140.59	57.72	218.58	3.79*	0.91
	mpdA	AN10021	1.24	0.70	0.44	0.58	1.32	0.56
Glycolysis	*hxkB*	AN2638	0.21	0.30	0.04	0.08	1.96	1.44
	hxkC	AN4255	41.34	36.24	15.95	21.93	1.37*	0.88
	hxkD	AN2180	80.91	92.70	18.34	32.42	1.77*	1.15

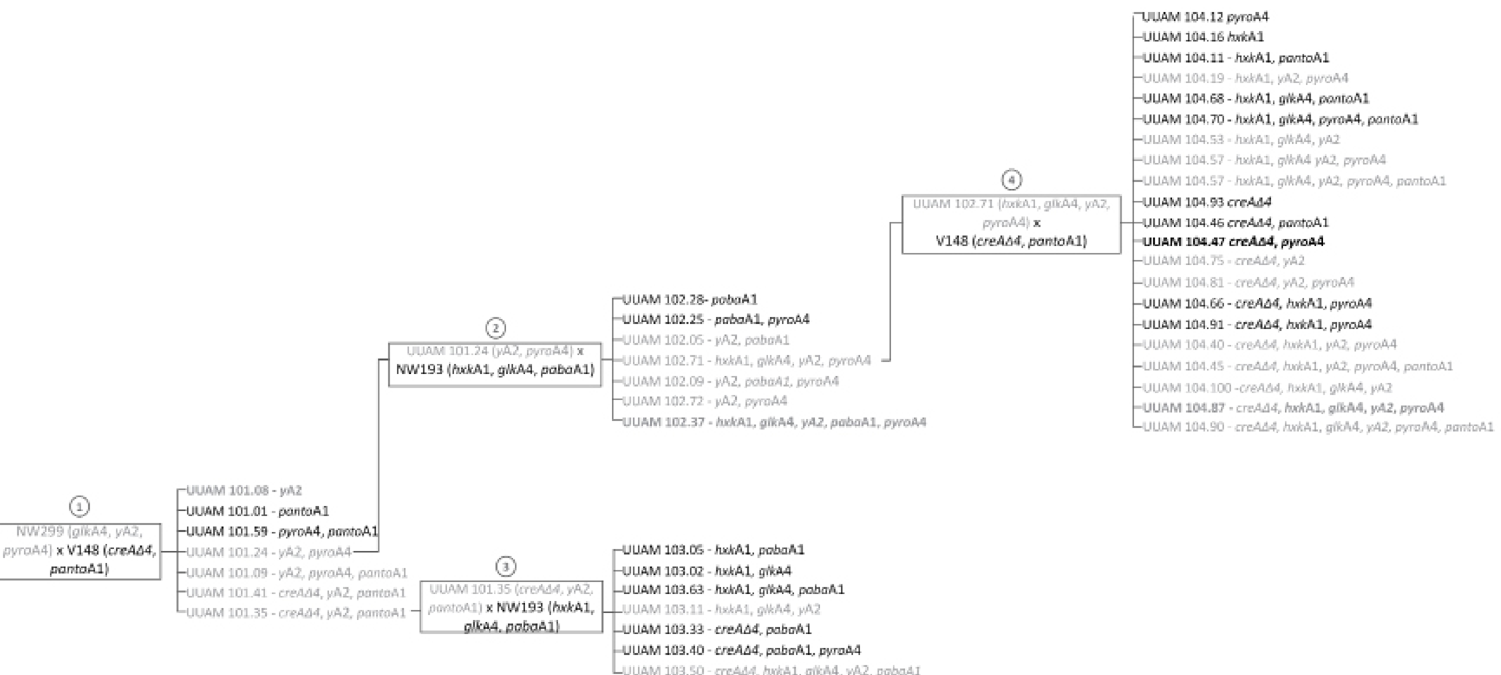

Figure S1 | Schema of sexual crossing in *A. nidulans.* The strains were crossed according to the schema. Strains resulting from cross 1-4 were named UU-AM101 – UU-AM104 respectively. We isolated progeny as separate colonies of the parental strains or recombinant genotypes. The strains used in this study are in bold, light grey color (*y*A2) and dark color (absence of *y*A2)

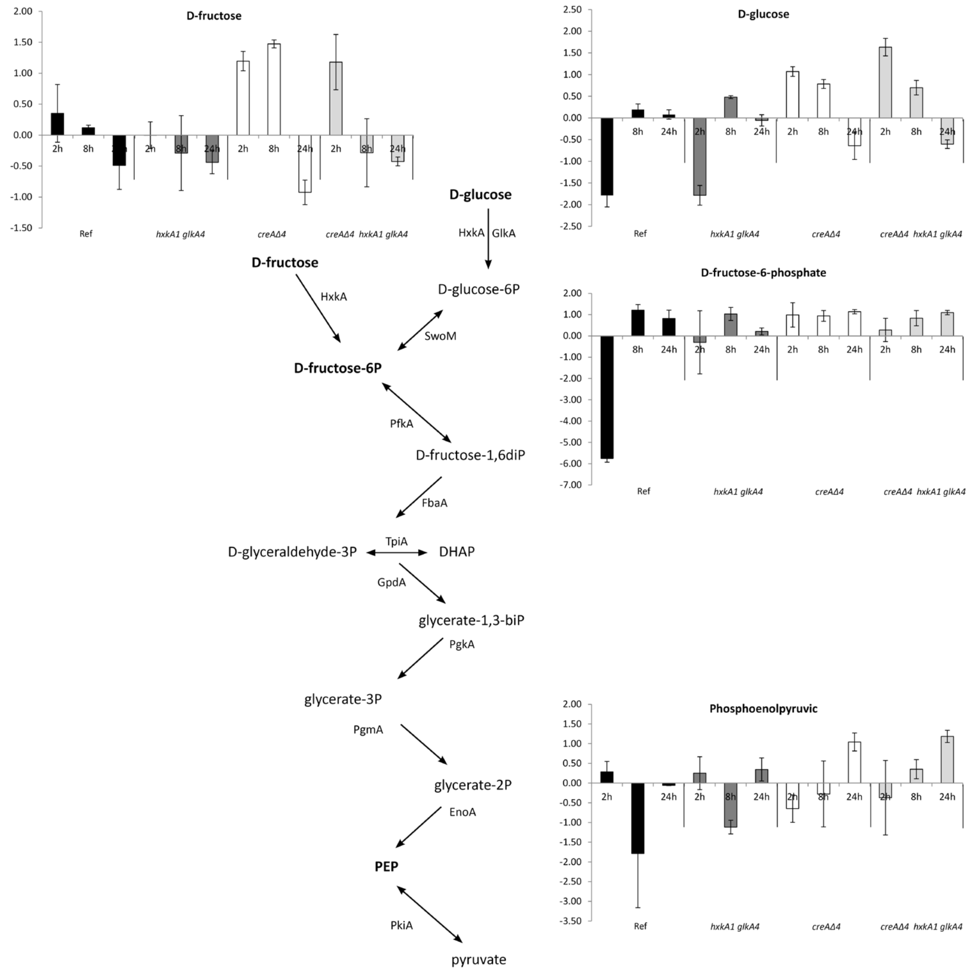

Figure S2 | Metabolomics quantification of selected metabolites from the glycolysis pathway.

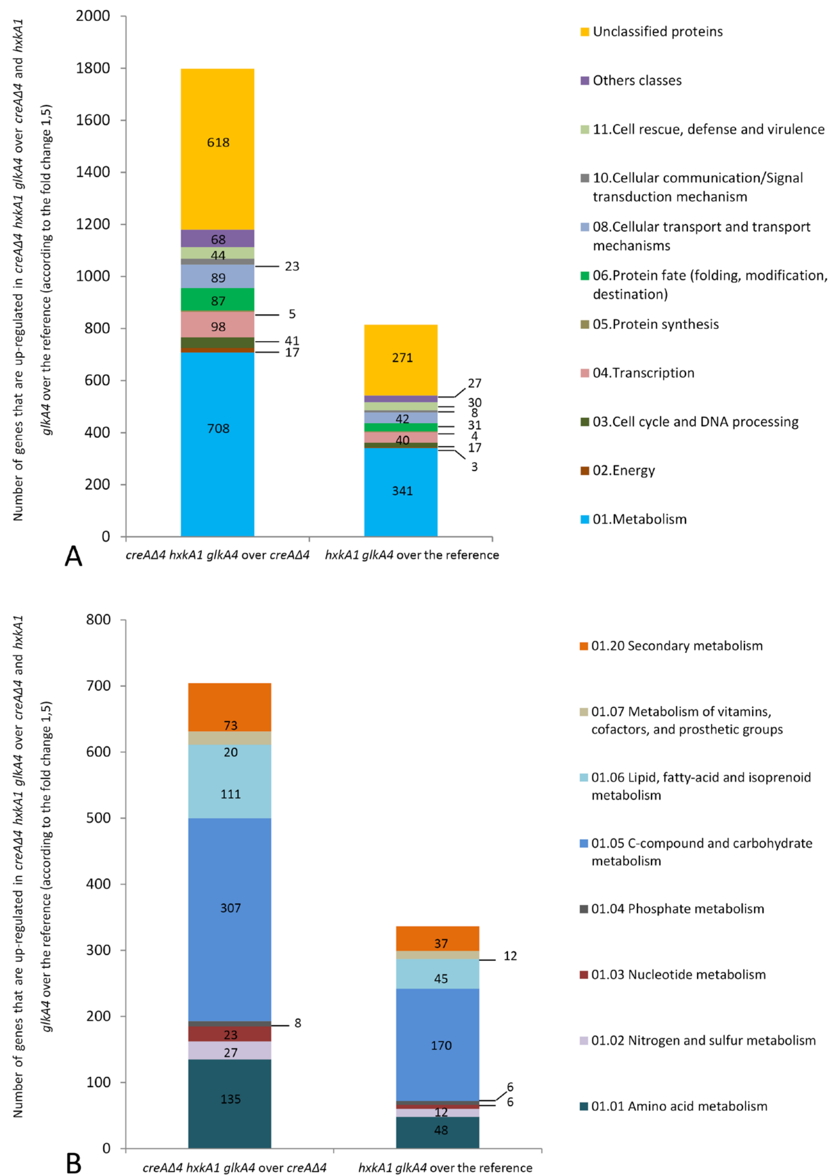

Figure S3 | Functional classification of *A. nidulans* genes according to FunCat. **(**A) FunCat classes are, 01, Metabolism; 02, Energy; 03, Cell cycle and DNA processing; 04, Transcription; 05, Protein synthesis; 06, Protein fate; 08, Cellular transport and transport mechanisms; 10, Cellular communication/signal transduction mechanism; 11, Cell rescue, defense and virulence; Others classes: (13, Regulation of/interaction with cellular environment; 14, Cell fate; 25, Development (systemic); 29, Transposable elements, viral and plasmid proteins; 30, Control of cellular organization; 40, Subcellular localization; 45, Tissue localization; 63, Protein with binding function or cofactor requirement; 67, Transport facilitation) and Unclassified proteins. The numbers represent the number of genes in each category. The lengths of the bar are proportional to the number of genes in each category. (B) Metabolism (01): amino acid metabolism (01.01); nitrogen and sulfur metabolism (01.02); nucleotide metabolism (01.03); phosphate metabolism (01.04); C-compound and carbohydrate metabolism (01.05); lipid, fatty-acid and isoprenoid metabolism (01.06); metabolism of vitamins, cofactors, and prosthetic groups (01.07); secondary metabolism (01.20).

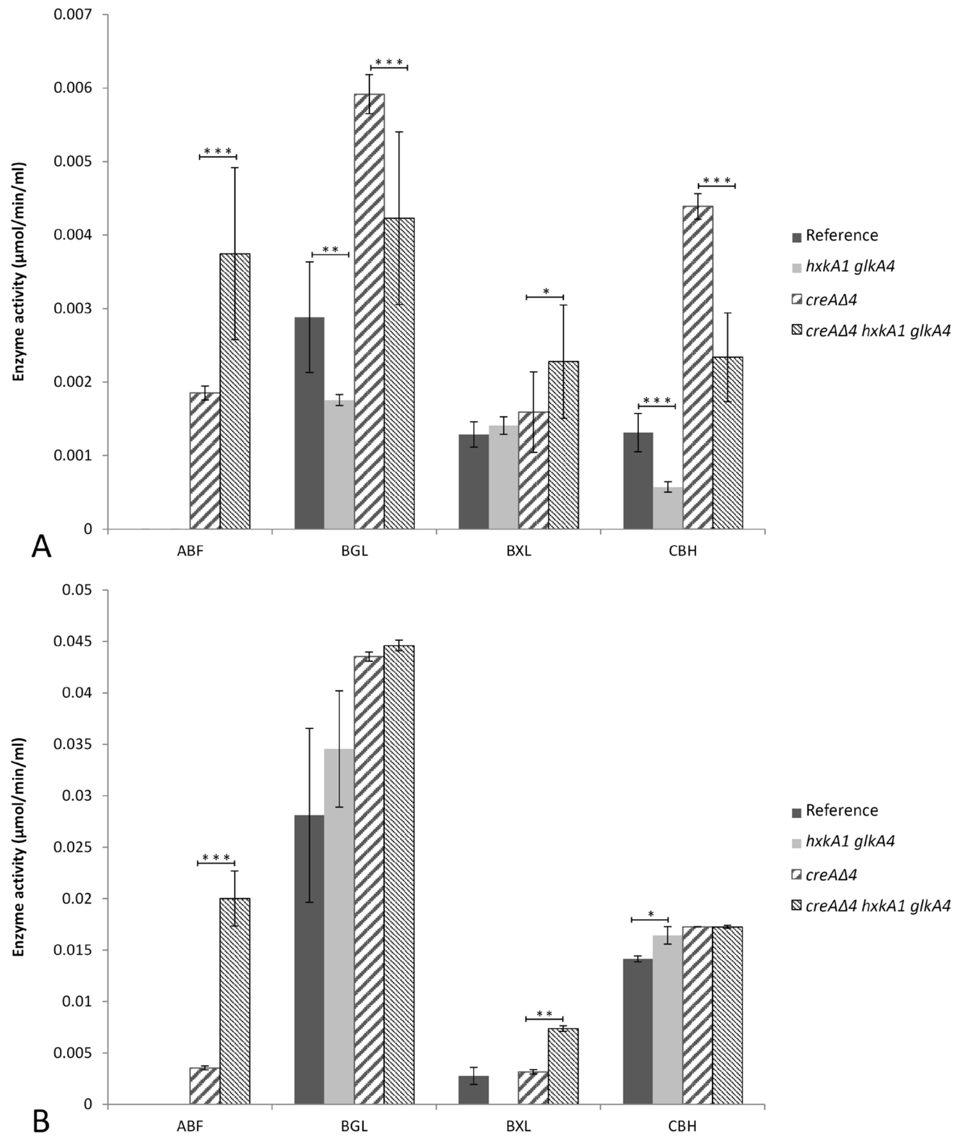

Figure S5 | Comparison of extracellular enzyme activities in the reference and disruption strains. Reference, *hxkA1 glkA4, creAΔ4* and *creAΔ4 hxkA1 glkA4 A. nidulans* strains were transferred for 8h (A) and 24h (B) in wheat bran. Enzyme activities of α-arabinofuranosidase (ABF), β-glucosidase (BGL), β-xylosidase (BXL) and cellobiohydrolase (CBH) in µmol pnp/ml/min/mg proteins. Means and SD (error bars) were calculated from two biological replicates with three technical replicates. Students t-test was performed between reference and the double mutant and between *creAΔ4* and the triple mutant. $p<0.005$ (***), $p<0.05$ (**), $p<0.05$ (*).

Chapter 7

CreA affects the physiology of *Aspergillus nidulans* during growth on plant biomass

Khosravi E, Battaglia E, Kun RS, Dalhuijsen S, Visser J, de Vries RP - in preparation

Abstract

Carbon catabolite repression (CCR) is a key regulation system found in most microorganisms. CCR is a process that ensures use of the energetically most favourable carbon source in an environment and represses the use of alternative carbon source when a better source is available.

In the filamentous fungus *Aspergillus nidulans* CCR is mediated by the transcription factor CreA, a C_2H_2 finger domain DNA-binding protein [1], that appears to be present in all fungi. Many studies on carbon repression in Aspergilli have been performed, but most focus on the role of CreA at high monosaccharide concentrations. However, in most natural biotopes, the concentration of monosaccharides is low.

In this study we evaluated the role of CreA during growth on a more natural substrate, wheat bran, which only contains low levels of free monosaccharides. We performed a transcriptome study, focusing in particular on CAZyme-encoding genes and central carbon metabolism as they have been shown to be highly sensitive to CreA repression.

The results showed that transcript levels of CAZy genes involved in degradation of cellulose, arabinoxylan and galactomannan and key central metabolic genes were affected in the *creAΔ4* mutant. Therefore, our results showed that CreA effects also occur at low monosaccharides levels. CreA influences fungal physiology already at low levels of free monosaccharides, suggesting an important role for this regulator in natural habitats of fungi.

Introduction

Filamentous fungi are characterized by a diverse metabolism and an extracellular enzyme spectrum that enables them to degrade a variety of polymeric carbon sources, such as cellulose, hemicellulose, pectin and lignin, and convert the resulting monomers. To ensure preferential use of the most favourable carbon source fungi possess a regulatory mechanism that results in the repression of the synthesis of enzymes required for the degradation of alternative carbon sources, when a preferred carbon source (e.g. glucose) is present in the environment. Early studies with the model fungus *A. nidulans* established that the carbon catabolite repression (CCR) is mediated by the transcription factor CreA [2-5], which encodes a C_2H_2-zinc finger protein that binds to a 5'-SYGGRG-3' target sequence [6-8].
CreA has been shown to be involved in many processes, such as the regulation of proline, ethanol, xylan and arabinan utilization in *A. nidulans* [1, 9, 10]. Studies in a related fungus, *A. niger*, revealed that CreA mediated repression not only occurs in the presence of glucose, but also is triggered by other monomeric carbon sources [11, 12].
The function of CreA is also subject to regulation at several levels; it has been shown that CreA and XlnR form a transcriptional complex regulating *A. nidulans* xylanolytic genes [13]. Similarly, in *A. nidulans* CreA and AlcR form a transcriptional cascade to control ethanol utilization [14]. Ries et al.,2016 [10] also showed that the cellular localization of CreA plays a key role in the expression of genes involved in the conversion of complex carbon sources and therefore has an important role in determining the growth on many carbon sources, where it mediates the expression of genes required for taking up, degrading and converting sugars into energy [10].
Past studies have mainly focused on the effect of CreA during growth on both monosaccharides and polysaccharides and little is known about how the repressor affects the physiology of *A. nidulans* during growth on plant biomass. In fact, in most natural biotopes, the concentration of monosaccharides is low and can therefore not be easily linked to most CreA studies using 1-3% of monosaccharide.
Wheat bran (WB) is a plant biomass substrate that contains different cell wall polysaccharides, such as cellulose, (arabino)xylan and xyloglucan, but also significant amounts of starch, lignin, mineral compounds and proteins [15]. During growth on wheat bran, the fungus secretes a wide range of Carbohydrate Active enZymes (CAZymes), which act in synergy to degrade the polysaccharides [16]. This study aims to gain a thorough understanding of the mechanism used by *A. nidulans* to degrade and grow on a complex lignocellulosic substrate. In this study we used a progeny of the mutant strain *creAΔ4* [17, 18]. The promotor, 5' untranslated, and a 702 bp fragment of the *creA* ORF, containing the zinc finger regions of *creA* were replaced by a 3091 bp insert containing the entire argB+ coding sequence. This replacement has been proved by sequencing. The mutant strain has been analysed on wheat bran with regard to growth and gene expression. The transcriptomics analysis was focused in particular on CAZymes involved in the plant biomass degradation.

Materials and Methods

Strains, media and growth conditions

The *A. nidulans* reference strain (CBS 141343, *y*A2) and *creA* mutant (CBS 141344, *creAΔ4*, *pyro*A4) were used in this study. CBS 141344 resulted from a cross between V148 (*creAΔ4, pantoA1*, a progeny of *creAΔ4* (*pabaA1, ya2, argB2)* [17] with UUAM 102.71(*hxkA1 glkA4, yA2, pyroA4*) (see Chapter 6). Both strains were grown in minimal medium (MM) or complete medium (CM) with addition of a carbon source [19].

For the production of spores, the strains were cultivated on plates containing CM with 1.5% (w/v) agar supplemented with 1% D-xylose and incubated at 37°C in the dark for 5 days. The spores were harvested in ACES buffer and counted with a haemocytometer (THOMA) under 40x magnifications. Ring plates of MM with 1% monosaccharide or 3% crude carbon source were used for growth profiling. The medium was supplemented with pyridoxine (pyro, 0.2 $g.l^{-1}$). The plates were inoculated with 2 µl spore solutions at $5x10^5$ spores/ml and incubated at 37°C during 3-4 days. For the transfer experiments, both strains were pre-grown in 1 L Erlenmeyer flasks that contained 200 ml of CM plus 2% D-xylose. Liquid cultures of two biological replicates were inoculated with 10^6 spores/ml and shaken at 250 rpm in a rotary shaker. After 16h, the mycelium was harvested by filtration, washed twice with MM and transferred to 250 ml Erlenmeyer flaks containing 50 ml MM plus 2% wheat bran and pyridoxine. The mycelium was harvested by vacuum filtration and culture samples were taken after 2, 8 and 24h of incubation. The mycelium samples were immediately dried between tissue paper and frozen in liquid nitrogen. For RNA analysis only 2h samples were used.

RNA extraction, cDNA library preparation and RNA-seq

Total RNA was extracted from mycelium ground in a Tissue Lyser (QIAGEN) using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. RNA integrity and quantity were analyzed on a 1% agarose gel using gel electrophoresis and with the RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA library preparation and sequencing reactions were performed by BGI Tech Solutions Co., Ltd. (Hong Kong). Illumina library preparation, clustering, and sequencing reagents were used throughout the process following the manufacturer's recommendations (http://illumina.com). On average 51 bp sequenced reads were obtained, producing approximately 570 MB raw data for each sample.

RNA-seq data analysis and functional annotation

Raw reads were produced from the original image data by base calling. After data filtering, the adaptor sequences, highly 'N' containing reads (> 10% of unknown bases) and low quality reads (more than 50% bases with quality value of < 5%) were removed. After data filtering, on average, ~95% clean reads remained in each sample. Clean reads were then mapped to the genome of *A. nidulans* (AspGD) using SOAPALIGNER/SOAP2 [20]. No more than two mismatches were allowed in the alignment. On average, 90% total mapped reads to the genome was achieved. The gene expression level was calculated by using the RPKM method [21]. Genes with expression value higher than 150 were considered highly expressed (approximately top 5%) and differential expression was identified by CyberT bayesian ANOVA algorithm [22] with a cut-off value of fold change > 1.5 and a p-value (corrected by multiple tests) < 0.05. The RNA-seq data have been submitted to Gene Expression Omnibus (GEO) [23] with accession number: GSE94775

Orthologous genes between *A. niger* CBS 513.88 and *A. nidulans* FGSC A4 were obtained from AspGD [24] and FunCat [25] functional annotation was mapped accordingly. Genes that had a fold-change of at least 1.5 between *creAΔ4* vs the reference strain were analyzed regarding the functional annotation.

Results

A dysfunctional CreA affects growth on monosaccharides, polysaccharides and plant biomass derived substrates

The reference CBS 141343 and the *creAΔ4* strain CBS141344 were used in this study. The *creA* mutant phenotype of the *creAΔ4* strain was confirmed in chapter 6. A growth profile of the strains on a selection of carbon sources was performed to analyze the effect of the *creAΔ4* mutant (Fig. 1). Four monosaccharides were selected and one crude substrate. On all carbon sources the *creAΔ4* strain had a reduced growth compared to the reference. Growth of the reference strain and *creAΔ4* strain was analyzed on 12 pure polysaccharides and untreated agricultural waste substrates described in chapter 6. On all the polysaccharides and crude substrates tested, the *creAΔ4* strain had a reduced growth compared to the reference. The data-set from chapter 6 was used to analyse the CreA effect in more detail during growth on wheat bran by *A. nidulans*.

Most of the up-regulated genes in the *creAΔ4* strain compared to the reference are assigned to the C-compound and carbohydrate metabolism class

RNA-sequencing of *A. nidulans* reference and *creAΔ4* strain grown for 2h after transfer to liquid medium containing wheat bran was performed to examine changes in gene expression. To classify the function of the predicted *A. nidulans* genes, the functional catalogue (FunCat) was used [25].

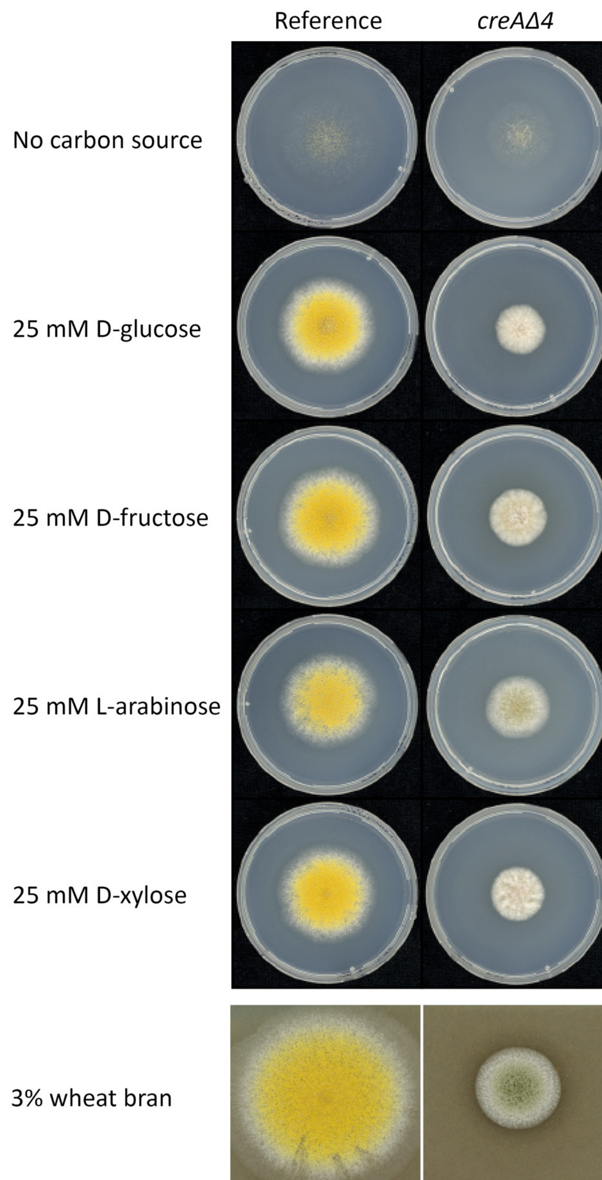

Figure 1 | Growth of the reference and *creAΔ4* strains on different carbon sources. The *A. nidulans* reference and *creAΔ4* strains were grown on agar (1.5%) plates using MM with 25 mM D-glucose, 25 mM D-fructose, 25 mM D-xylose, 25 mM L-arabinose and 3% wheat bran for 3 days at 37°C. Spore inoculations were done with 1000 spores.

Out of the total 10460 genes predicted in the genome of *A. nidulans*, 5812 were associated with FunCat (component, function, and process) and were assigned to 19 main groups (see legend Fig. 2). 977 genes were up-regulated (fold change ≥ 1,5 and p-value <0.05) in the *creAΔ4* mutant compared to the reference.

Metabolism was the major class with 26%, together with the Unclassified Proteins (30%), followed by Protein synthesis (12%), Transcription (10%) and Protein fate (8%) (Fig.2A). The Metabolism class contained 8 subgroups (see legend Fig. 2), of which C-compound and carbohydrate metabolism (C-CM) accounted for the largest number of up-regulated genes (51%, 130 genes) (Fig. 2B). In the C-CM subgroup 38 genes (29%) were CAZy genes involved in plant biomass degradation and 10 genes (8%) were metabolic genes involved in sugar catabolism.

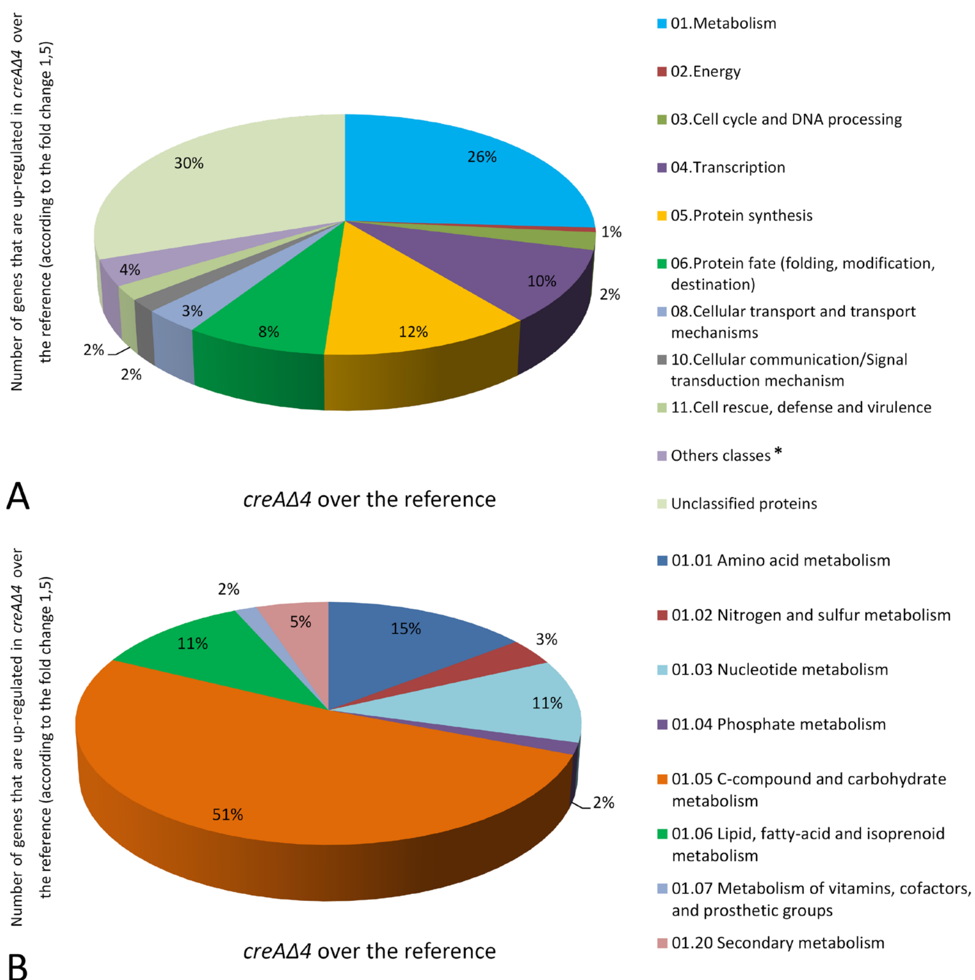

Figure 2 | Functional classification of *A. nidulans* genes according to FunCat. The numbers represent the percentage in each category. (A) Analysis of the FunCat classes. *Others classes: (13, Regulation of/interaction with cellular environment; 14, Cell fate; 25, Development (systemic); 29, Transposable elements, viral and plasmid proteins; 30, Control of cellular organization; 40, Subcellular localization; 45, Tissue localization; 63, Protein with binding function or cofactor requirement; 67, Transport facilitation). (B) Detailed analysis of the Metabolism class.

Cellulases and xylanases were up-regulated in the *creAΔ4* strain in wheat bran

Our analysis was focused on the cellulose and (hemi)cellulose-active CAZymes due to the high amount of cellulose and xylan in wheat bran. The detailed list of all the CAZymes involved in the plant biomass degradation can be found in Table S1.

These CAZymes were divided based on their enzyme class (Table 1). In this paragraph, we highlighted genes that are up-regulated (fold change ≥ 1,5, p value<0.05 and FPKM>20) and those were analyzed. The cellulose sub-group included β-1,4-endoglucanases (EGL), cellobiohydrolases (CBH), cellobiose dehydrogenase (CDH) and β-glucosidase (BGL). Two CBHs (*cbhA*, c*bhD*), one CDH (AN7230), three EGLs (*eglA*, *eglB* and AN8068) and five BGLs (*bglI*, *bglR*, *bglL*, *bglA* and *bglH*) were up-regulated in the *creAΔ4* strain compared to the reference (Table 1). The xylan sub-group involved β-1,4-endoxylanase (XLN), β-1,4-xylosidase (BXL), acetyl xylan esterase (AXE), arabinoxylan arabinofuranohydrolase (AXH) and α-glucuronidase (AGU). Three XLNs (*xlnC*, *xlnA* and *xlnE*), five BXLs (*bxlC*, *xlnD*, AN1477, AN7275 and AN8477) one AXE (*axeA*), two AXH (*axhA* and *axhB*) and one AGU (*aguA*) were up-regulated in the *creAΔ4* strain (Table 1). The galactomannan sub-group contained β-1,4-endomannanase (MAN), β-1,4-mannosidase (MND) and α-1,4-galactosidase (AGL). Four MANs (*manC*, *manB* and *manA*), one MND (*mndB*) and three AGLs (*aglG*, *aglD* and *aglC*) were up-regulated in the *creAΔ4* strain. The xyloglucan sub-group included 1,4-endoglucanase (XEG), α-xylosidase (AXL) and α-fucosidase (AFC). On average the five xyloglucanases were lowly expressed in both stains (Table S1). The starch sub-group contained α-amylase (AMY), glucoamylase (GLA) and α-glucosidase (AGD). Three AMYs (*amyB*, *amyF* and *amyG*), one GLA (*glaB*) and three AGD (*agdB*, *agdF* and *agdE*) were up-regulated in the *creAΔ4* strain (Table 1).

Also, few enzymes involved in the degradation of the pectin were affected by the *creA* deletion. One pectin methyl esterase (PME; *pmeB*), one rhamnogalacturonan acetyl esterase (RGAEA; *rgaeA*), two exopolygalacturonases (PGX; *pgxA* and *pgxB*), one endo-α -L-arabinosidase (ABN; *abnA*), one exo-arabinanase (ABX; *abxA*), two unsaturated rhamnogalacturonan hydrolases (URH; *urhA* and *urhC*), two pectin lyases (PEL; *pelA* and *pelB*), two pectate lyase (PLY; *plyC* and *plyF*) and one rhamnogalacturonan lyase (RGL; *rglA*) were up-regulated in the *creAΔ4* strain (Table S1).

The last sub-group we were interested in, CAZymes with broad specificity. This group contained enzymes that act on various polysaccharides such as α-arabinofuranosidase (ABF), feruloyl esterase (FAE), β-1,4-galactosidase (LAC) and lytic polysaccharide monooxygenase (LPMO). These enzymes are required for complete degradation of cellulose, xylan and xyloglucan which are present in wheat bran. After 2h of transfer in wheat bran, four ABFs (*abfB*, *abfC*, AN2533 and AN7781), one FAE (*faeC)* and six LPMO were up-regulated in the *creAΔ4* compared to the reference (Table 1).

Table 1 | CAZy genes involved in the degradation of plant biomass in *A. nidulans* that are significantly up-regulated.
The gene with expression higher than 150 are considered highly expressed and marked dark grey. The genes with expression lower than 20 are considered low expressed and marked light grey. The comparison between strains is *creAΔ4* over the reference. The cut-off for differential expression is fold change >1,5.

Substrate	Enzyme class	Enzyme code	Abbrv.	Gene number	Reference	*creAΔ4*	*creAΔ4*/Ref
Cellulose	Cellobiohydrolase	CBH	*cbhA*	AN5176	2.91	15861.90	5454.602
Cellulose	Cellobiohydrolase	CBH	*cbhD*	AN1273	9.02	5280.49	585.594
Cellulose	Putative cellobiose dehydrogenase	CDH		AN7230	1.03	657.11	636.501
Cellulose	Endo-β-1,4-glucanase A	EGL	*eglA*	AN1285	8.80	9937.98	1129.844
Cellulose	Endo-β-1,4-glucanase	EGL	*eglC*	AN3418	19.24	4020.54	208.943
Cellulose	Putative β-1,4-endoglucanase	EGL		AN8068	9.58	35.15	3.670
Cellulose	Endo-β-1,4-glucanase	EGL	*eglB*	AN5214	2.03	3.68	1.812
Cellulose	Putative β-glucosidase	BGL	*bglI*	AN2227	20.20	825.98	40.890
Cellulose	Putative β-glucosidase	BGL	*bglR*	AN9183	22.94	107.07	4.668
Cellulose	β-glucosidase	BGL	*bglL*	AN2828	42.86	134.83	3.146
Cellulose	Putative β-glucosidase	BGL	*bglA*	AN4102	344.16	953.20	2.770
Cellulose	Putative β-glucosidase	BGL	*bglH*	AN3903	14.74	30.30	2.056
Xylan	β-1,4-endoxylanase	XLN	*xlnC*	AN1818	86.05	11654.05	135.436
Xylan	β-1,4-endoxylanase	XLN	*xlnA*	AN3613	88.19	5091.04	57.731
Xylan	β-1,4-endoxylanase	XLN	*xlnB*	AN9365	3.82	96.37	25.255
Xylan	Putative β-1,4-endoxylanase	XLN	*xlnE*	AN7401	1.59	7.21	4.535
Xylan	Putative β-1,4-xylosidase	BXL		AN1477	72.87	1270.83	17.440
Xylan	Putative β-1,4-xylosidase	BXL		AN7275	6.85	57.46	8.385

Xylan	β-xylosidase / α-L-arabinofuranosidase	BXL	*bxlB*	AN8401	0.44	3.48	7.936
Xylan	Putative β-1,4-xylosidase	BXL		AN8477	12.27	72.37	5.899
Xylan	β-xylosidase	BXL	*xlnD*	AN2359	408.81	1838.65	4.498
Xylan	Putative β-1,4-xylosidase	BXL	*bxlC*	AN2217	12.03	49.79	4.140
Xylan	Putative β-1,4-xylosidase	BXL		AN6751	2.96	7.96	2.694
Xylan	Acetyl xylan esterase	AXE	*axeA*	AN6093	15.43	2352.48	152.492
Xylan	Putative α-L-arabinofuranosidase	AXH	*axhB*	AN2632	3.08	2401.56	779.634
Xylan	α-L-arabinofuranosidase	AXH	*axhA*	AN7908	85.97	1542.05	17.936
Xylan	α-1,2-glucuronidase	AGU	*aguA*	AN9286	32.20	282.30	8.768
Galactomannan	Endo-β-1,4-mannanase	MAN	*manC*	AN6427	4.05	395.72	97.707
Galactomannan	Endo-β-1,4-mannanase	MAN	*manB*	AN3297	3.37	190.32	56.489
Galactomannan	Endo-β-1,4-mannanase	MAN	*manA*	AN3358	0.85	24.23	28.637
Galactomannan	β-mannanase	MAN		AN6833	0.10	1.12	10.667
Galactomannan	Endo-β-1,4-mannanase	MAN	*manE*	AN7413	0.22	0.99	4.414
Galactomannan	β-mannosidase	MND	*mndB*	AN3368	174.07	1531.70	8.800
Galactomannan	Putative α-galactosidase	AGL	*aglG*	AN9035	9.42	104.42	11.083
Galactomannan	α-galactosidase	AGL	*aglD*	AN7152	27.44	54.69	1.993
Galactomannan	α-galactosidase	AGL	*aglC*	AN8138	415.07	635.61	1.531
Starch	α-amylase	AMY	*amyB*	AN3402	0.41	994.93	2409.844
Starch	Putative α-amylase	AMY	*amyF*	AN3388	18.62	1634.47	87.787
Starch	Putative α-amylase	AMY	*amyG*	AN3309	10.69	26.41	2.470
Starch	Putative glucoamylase	GLA	*glaB*	AN7402	0.68	20.47	30.201
Starch	α-glucosidase	AGD	*agdB*	AN8953	371.89	5035.81	13.541

Starch	Putative α-glucosidase	AGD	*agdF*	AN10420	6.72	27.29	4.061
Starch	α-glucosidase	AGD	*agdE*	AN0941	35.27	89.86	2.547
Various substrates	α-L-arabinofuranosidase	ABF	*abfB*	AN1571	13.42	209.14	15.582
Various substrates	Putative α-L-arabinofuranosidase	ABF		AN2533	49.07	585.76	11.938
Various substrates	Putative α-L-arabinofuranosidase	ABF		AN8472	1.43	11.41	7.965
Various substrates	α-L-arabinofuranosidase	ABF	*abfC*	AN1277	197.34	717.92	3.638
Various substrates	Putative α-L-arabinofuranosidase	ABF		AN7781	178.04	582.74	3.273
Various substrates	Feruloyl esterase	FAE	*faeC*	AN5267	151.55	1790.23	11.813
Various substrates	Putative β-galactosidase	LAC	*lacD*	AN3201	1.85	13.66	7.385
Various substrates	Putative β-galactosidase	LAC		AN2804	4.23	17.39	4.114
Various substrates	β-galactosidase	LAC	*lacA*	AN0756	8.44	18.14	2.149
Unknown	Lytic polysaccharide monooxygenase	LPMO		AN3860	15.66	22716.85	1450.242
Starch?	Lytic polysaccharide monooxygenase	LPMO		AN6103	5.12	1031.39	201.402
Unknown	Lytic polysaccharide monooxygenase	LPMO		AN6428	2.98	103.84	34.798
Unknown	Lytic polysaccharide monooxygenase	LPMO		AN1602	1.75	47.57	27.175
Unknown	Lytic polysaccharide monooxygenase	LPMO		AN3046	5.23	97.92	18.730
Unknown	Lytic polysaccharide monooxygenase	LPMO		AN9524	0.44	3.16	7.102
Unknown	Lytic polysaccharide monooxygenase	LPMO		AN1041	26.25	84.69	3.226

A dysfunctional CreA affects the gene expression of metabolic genes involved in the conversion of hexoses and pentoses

Wheat bran consists of D-glucose, D-xylose, L-arabinose and small amounts of D-galactose and uronic acids (see chapter 6 for the detailed composition). Therefore, our transcriptomic analysis focused on glycolysis, citric acid cycle (TCA), pentose phosphate pathway (PPP) and the pentose catabolic pathway (PCP). In the glycolysis, glucose-6P-isomerase (*swoM*), glyceraldehyde-3P dehydrogenase (*gpdA)* and pyruvate kinase (*pkiA*) were significantly up-regulated in the *creAΔ4* compared to the reference (Fig.3; Table S2), whereas glucokinase (*glkA*), phosphofructokinase (*pfkA*), fructose-biphosphate aldolase (*fbaA*) and triose-phosphate isomerase (*tpiA*) were significantly down-regulated in the *creAΔ4* compared to the reference. In the TCA cycle, the putative citrate synthase (*citA*), oxoglutarate dehydrogenase (*kgdA*) and putative succinate-CoA ligase (*scsB*) were up-regulated in the *creAΔ4* compared to the reference, while most of the other genes involved in the TCA cycle were down-regulated in the *creAΔ4* strain (Fig. 3; Table S2). In the D-mannose pathway, the expression profiles differed, mannose-6P isomerase (*pmiA*) was up-regulated in *creAΔ4*, phosphomannomutase (*pmmA*) was down-regulated in the *creAΔ4* strain compared to the reference strain. In the PCP, L-arabitol dehydrogenase (*ladA*), L-xylo-3-hexulose reductase (*lxrA*) and D-xylose reductase (*xyrA*) were up-regulated in *creAΔ4* compared to the reference, while arabinose reductase (*larA*) was down-regulated in the *creAΔ4* strain (Fig.4). In the PPP, D-ribulose-3P epimerase (*rpeA*), transaldolase (*pppA*) and glucose-6P-1-dehydrogenase (*gsdA*) were up-regulated in the *creAΔ4* compared to the reference, while D-ribulokinase (*rbtA*), ribokinase (*rbkA*) and ribose-5P isomerase (*rpiB*) were down-regulated in the *creAΔ4* (Fig.4). The expression of most of the genes of the PPP was high in the reference and *creAΔ4* strain.

Wheat bran contains also other monosaccharides such as galactose and uronic acid. However, genes involved in the Leloir pathway, oxido-reductive D-galactose pathway and D-galacturonic acid pathway did not show a clear effect in the *creAΔ4* mutant. In the Leloir and oxido-reductive pathway, the majority of the genes were down-regulated in the *creAΔ4* strain compared to the reference, except sorbitol dehydrogenase (*sdhA*) which was up-regulated in the *creAΔ4* strain (Fig. S1). In the D-galacturonic-acid pathway, D-galacturonic acid reductase (*gaaA*), 2-keto-3-deoxy-L-galactonate aldolase (*gaaC*) and L-glyceraldehyde reductase (*gaaD*) were down-regulated in the *creAΔ4* strain compared to the reference (Fig. S1 and Table S2).

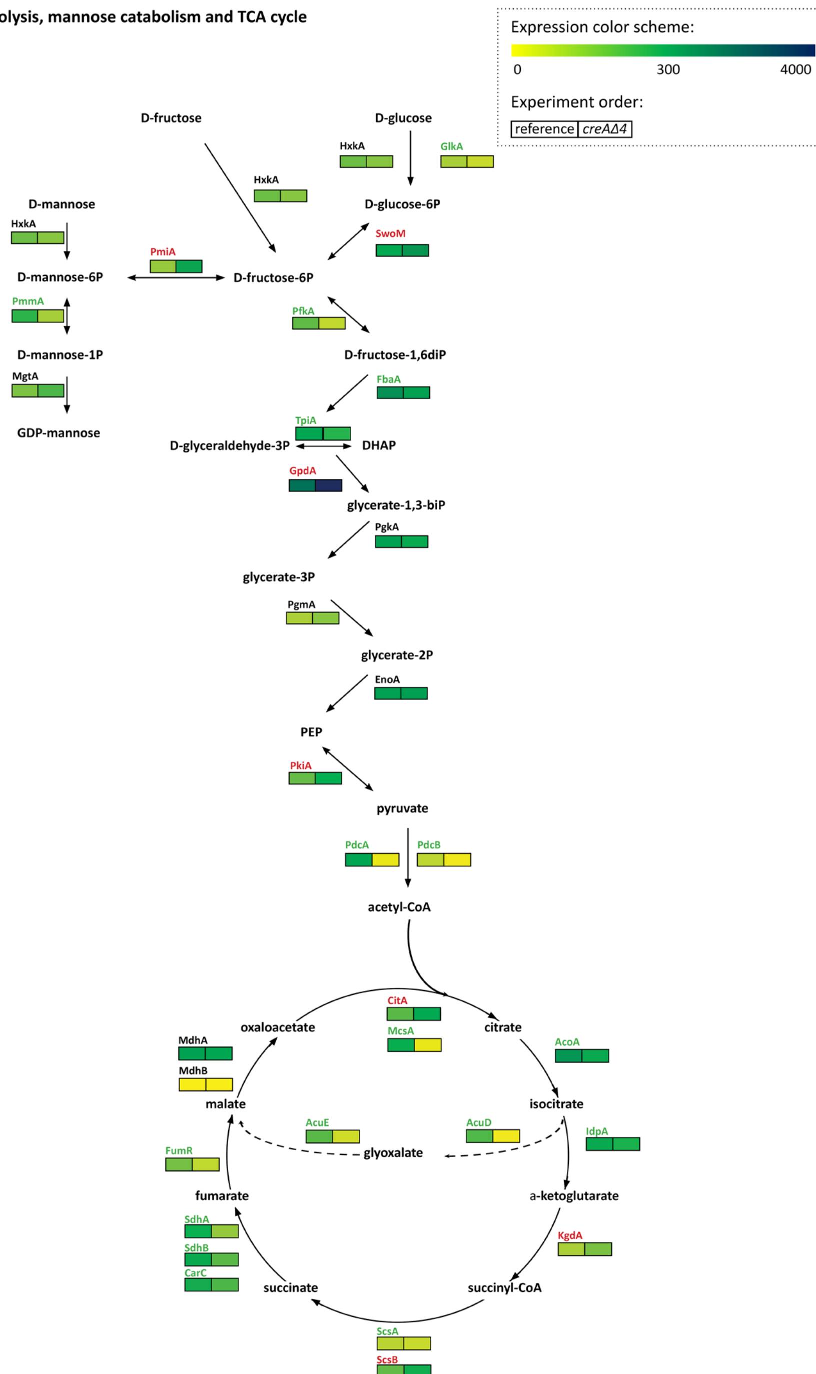

Figure 3 | Schematic representation of the expression of genes involved in the glycolysis in *A. nidulans* reference and *creAΔ4* strains, after 2h from D-xylose to liquid medium containing 1% (w/v) wheat bran. Gene expression values are presented under the genes and indicated by a color gradient. A decrease in expression is indicated by yellow squares and increased expression is indicated by a dark blue square. Up-regulated genes in are in red and down-regulated genes are in green.

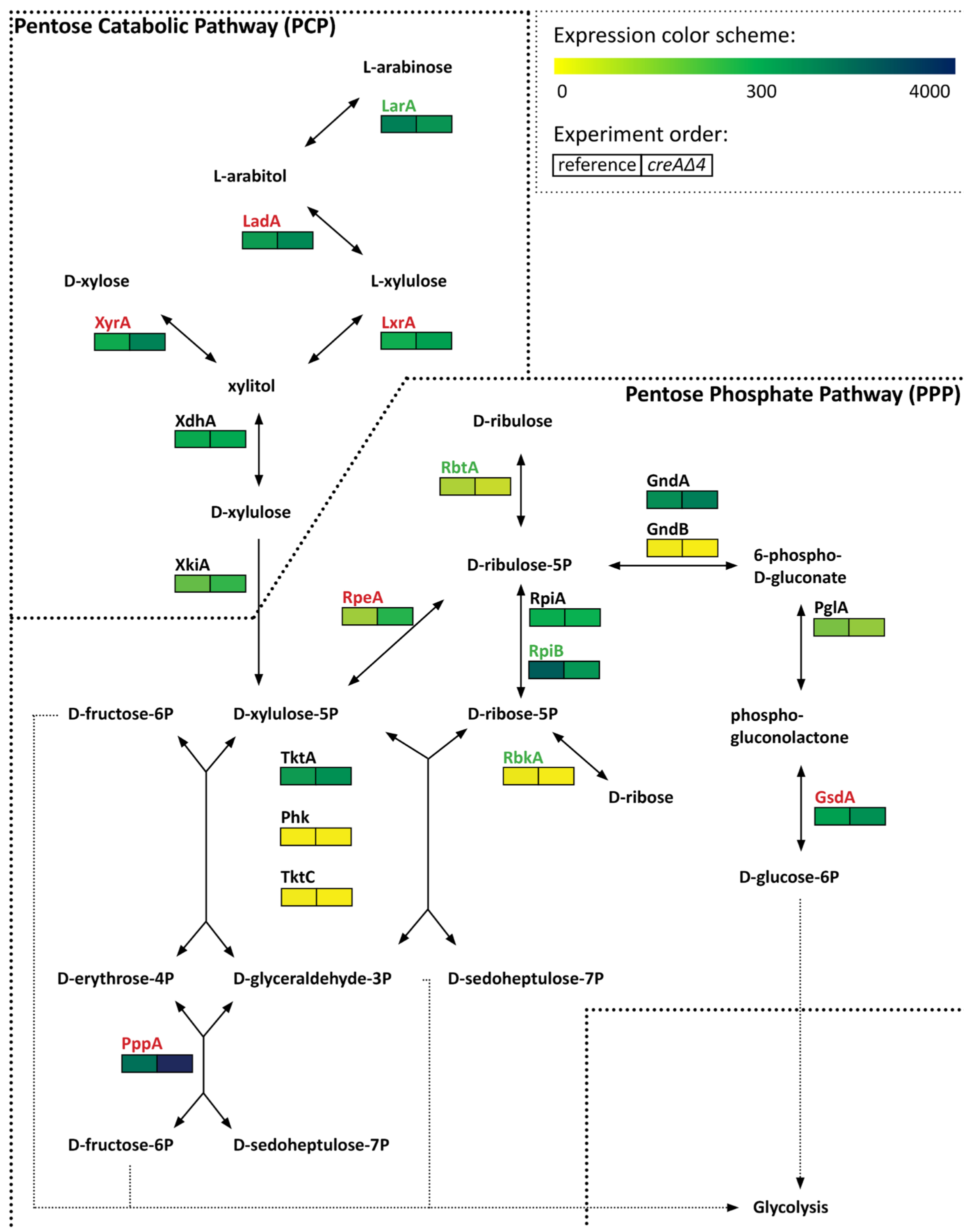

Figure 4 | Schematic representation of the expression of genes involved in the pentose phosphate pathway and the pentose catabolic pathway in *A. nidulans* reference and *creAΔ4* strains, after 2h from D-xylose to liquid medium containing 1% (w/v) wheat bran. Gene expression values are presented under the genes and indicated by a color gradient. A decrease in expression is indicated by yellow squares and increased expression is indicated by a dark blue square. Up-regulated genes in the *creAΔ4* strain are in red and down-regulated genes are in green.

ClrA and AmyR gene expression were affected in the *creA*Δ*4* strain

To analyse in more detail the *creAΔ4* mutant on wheat bran, expression of other characterized transcription factors (TFs) involved in the plant biomass degradation was also analysed (Table 2). ClrA has been identified in *Neurospora crassa* as a TF involved in the cellulose degradation [27]. In the *creAΔ4*, *clrA* was significantly up-regulated compared to the reference. AmyR is a TF that controls the genes involved in starch degradation. In *creAΔ4*, *amyR* was significantly up-regulated compared to the reference strain. Also, the repressor GaaX was down-regulated in the *creAΔ4* compared to the reference. Both D-galactose TFs were down-regulated in *creAΔ4* compared to the reference. The glucose-sensing regulator BglR/Col26 was as well down-regulated in the the *creAΔ4*.

Table 2 | Expression of known regulators genes involved in the plant biomass degradation in *A. nidulans*.
The gene with expression higher than 150 are considered highly expressed and marked dark grey. The genes with expression lower than 20 are considered low expressed and marked light grey. The comparison between strains is *creAΔ4* mutant over the reference. The cut-off for differential expression is fold change >1,5 (cells marked dark grey if up-regulated and light grey if down-regulated) and p-value <0.05 (*).

[a]Abbrv.	Gene number	Reference	*creAΔ4*	*creAΔ4*/Ref
XlnR	AN7610	21.81	27.08	1.24*
CreA	AN6195	507.49	37.69	0.07*
ClrA	AN5808	27.96	108.59	3.88*
AmyR	AN2016	68.04	102.10	1.50*
GalR	AN10550	3.42	0.34	0.10*
GalX	AN10543	7.06	2.53	0.36*
ClrB	AN3369	38.69	50.15	1.30*
InuR	AN3835	8.58	7.61	0.89
AraR	AN0388	24.01	22.19	0.92
GaaR	AN10548	30.97	43.05	1.39*
GaaX	AN10544	55.97	84.40	1.51*
Clbr	AN6091	73.25	20.61	0.28*
Clbr2	AN8125	30.50	18.33	0.60*
Clbr3	AN12148	7.49	2.71	0.36*
McmA	AN8676	229.68	145.95	0.64*
Ace1	AN2919	579.94	481.06	0.83
BglR/Col26	AN10423	4.01	1.39	0.35*

[a]List of regulator genes based on Benocci et *al.,* 2017 [28].

7

Discussion

One critical point in the conversion of lignocellulosic plant biomass to biofuels is the repression of genes encoding lignocellulose-degrading enzymes, when a preferred carbon source (e.g. glucose) is detected by the cell. In *A. nidulans* the CCR is mediated by the transcription factor CreA. Repression by CreA may be caused by different carbon sources, but glucose is probably the most repressive [1]. CreA ensures that the presence of the preferred carbon source prevents expression of genes involved in utilization of less-preferred carbon sources [13]. The role of CreA in the CCR was extensively studied using simple and pure polysaccharides, but the role of this transcription factor on a crude substrate such as wheat bran was less studied. [10]. The aim of this work was to study the *creAΔ4* mutant at the transcriptomic level, focusing in particular on CAZyme-encoding genes and central carbon metabolism as they have been shown to be sensitive to CreA repression [11, 29].

As shown before [17] and in Chapter 6, colony morphology of the *creAΔ4* mutant is affected on agar plates with both repressing and non-repressing monosaccharides. In chapter 6, the small and compact colony morphology of the *creAΔ4* strain was also observed on agar plates with different pure polysaccharides and crude substrates. This implies that a functional CreA protein is essential to ensure normal growth on substrates derived from plant biomass.

By comparing the RNA-seq data between the reference and the *creAΔ4* strain after 2h of transfer to wheat bran, we aimed to analyse the expression of genes required to degrade this substrate in this chapter. The functional enrichment analysis of the up-regulated genes using FunCat analysis showed that the Metabolism class was overrepresented, where 51% of the genes in this class were identified as being part of the C-CM subgroup. Overall, in all the up-regulated genes in the C-CM class, genes related to cellulose degradation (EGL and CBH) were among the genes that increased significantly in expression level in the *creAΔ4* strain. Then, we did a targeted analysis focused on the cellulose and (hemi)cellulose-active CAZymes due to the high amount of cellulose and xylan present in wheat bran. Several EGL, XLNs and BXL have been shown to be under carbon catabolite repression in *A. niger* (*xlnB*, *xlnD*) and *A. nidulans* (*eglA, xlnA*) [10, 30]. In our RNA-seq results, four XLNs (*xlnA*, *xlnB*, *xlnC* and *xlnE*) and seven BXLs (*bxlB*, *bxlC*, *xlnD*, AN1477, AN7275, AN8477 and AN6751) were significantly up-regulated in the *creAΔ4* strain. These results confirmed that CreA affects the expression of genes encoding functions involved in degradation of cellulose and xylan. In addition, most of the enzymes involved in the galactomannan degradation were also up-regulated in the *creAΔ4*, suggesting that the CreA is affecting not only the expression of cellulase- and xylanase-encoding genes but also that of galactomannan-related genes.

Interestingly, amylolytic genes were up-regulated in the *creAΔ4* strain. D-glucose or maltose present in wheat bran might act as an amylase inducer, as previously reported [31], triggering the nuclear localization of AmyR. AmyR is well characterized in *A. nidulans* [32]. Deletion of *amyR* in *A. nidulans* leads to significantly decreased amylolytic enzyme activities and restricted growth on both starch and maltose [33]. In our data set, AmyR was up-regulated in the *creAΔ4* strain compared to the reference strain.

This result is in accordance with the strong up-regulation of *amyB* and *amyF* in the *creAΔ4* strain. In *N. crassa*, another transcription factor COL-26 was shown to function synergistically with CRE-1 (CreA, in *A. nidulans*), in the CCR and in the regulation of cellulase gene expression [34]. A recent study showed that the cellulose-specific genes were among the genes that increased in expression in the Δ*col*-26 mutant on maltose and amylose [35]. The absence of Col-26 led to a decrease in expression of amylolytic genes. Therefore, in this study an antagonizing effect was suggested between activation of amylolytic genes versus cellulase genes, which is mediated by COL-26/AmyR. However, the ortholog of Col26 in *A. nidulans* was down-regulated in the *creAΔ4* strain. From these data we hypothesized that a regulatory interplay between CreA/AmyR/Col-26 might play a role in the cellulolytic and amylolytic gene expression in *A. nidulans* as well, indicating a much more complex regulatory system when the fungus is exposed to a crude substrate.

Another transcription factor involved in the cellulose degradation, ClrA, was significantly up-regulated in the *creAΔ4* strain compared to the reference. This regulator has been fully characterized in *N. crassa* (CLR-1/2) and partially in *A. nidulans* (ClrA) [27, 36]. Selected cellulases were shown to be controlled by ClrA and ClrB in the study of Coradetti et al., [27]. Two CBHs (*cbhA* and c*bhD*) were strongly up-regulated in the *creAΔ4* strain. These results confirm that CreA plays an important role in the utilization of complex substrates by increasing gene expression of genes encoding cellulose and arabinoxylan degrading enzymes.

In chapter 6, HPAEC-PAD sugar analysis was performed after 2, 8 and 24h of transfer to wheat bran. Glucose, xylose and arabinose accumulated after 2h in the *creAΔ4* strain compared to the reference strain and then levels reduced after 8 and 24 h of transfer to wheat bran in the *creAΔ4* strain. Together with the RNA-seq results, it suggests that glucose, xylose and arabinose release may be at much higher levels at the 2 h time-point than uptake in the *creAΔ4* strain.

The main catabolic pathways for the degradation of hexoses and pentoses were analyzed in this study. In the glycolysis, glucose-6P represents an important branch point to distribute the metabolic flux over glycolysis, PPP and the Leloir pathway. In our RNA-seq data, *swoM*, which converts glucose-6P to fructose-6P in a reversible reaction, was significantly up-regulated in the *creAΔ4* strain. Also, *gpdA*, which converts D-glyceraldehyde-3P into glycerate-1,3-biP and *pkiA*, which converts phosphoenolpyruvate (PEP) into pyruvate were up-regulated in the *creAΔ4* strain compared to the reference. The increase observed in *gpdA* expression might be due to an increased flux through the oxidative part of the PPP. It has been shown that when the *creAΔ4* strain was grown on a mixture of D-glucose and D-xylose, this part of the PPP was more active than on D-glucose alone [37]. In *A. nidulans*, the pyruvate arises from PEP which is generated via the last part of glycolysis [38]. Most of the genes in the TCA cycle were down-regulated in the *creAΔ4* strain, except *citA*, *kgdA* and *scsB*. The TCA cycle might be less active in the *creAΔ4*, and therefore pyruvate may only be derived from PEP. It has also been shown that the flux through the TCA cycle decreased in the *creAΔ4* when D-xylose was added to glucose medium [37].

The catabolism of pentoses includes NADPH reduction of the pentose to xylitol and the NAD+-dependent oxidation of the polyol to xylulose. Besides the up-regulation of genes of the oxidative part of the PPP, an important response is also observed in the non-oxidative PPP in the *creAΔ4* mutant which showed an increased expression of *rpeA* encoding the epimerase responsible for converting D-xylulose-5-phosphate into D-ribulose-5-phosphate and the *pppA* gene encoding a transaldolase. Xylose is the second most abundant monosaccharide present in wheat bran, therefore the demand for NADPH is increased and additional NADH is generated from the xylitol dehydrogenase (*xdhA*) step which converts D-xylitol into D-xylulose. This higher demand of NADPH seemed to be met by an increased flux through the PPP as shown previously [37].
We also looked at the catabolism of D-galactose and D-galacturonic-acid present in small amount in wheat bran. Most of the genes involved in the catabolism of those monosaccharides were down-regulated in the *creAΔ4* strain compared to the reference, maybe due to an increase of NADH demand from the PPP. The changes observed in transcript levels of key central metabolic genes in the *creAΔ4* strain that may result in dysregulation of the relative flux distributions.

To summarize, we have analyzed the transcriptomics response of the *A. nidulans creAΔ4* strain after a 2 h transfer to wheat bran, through the analysis of CAZy genes and central carbon metabolic genes. We observed that transcript levels of CAZy genes involved in degradation of cellulose, arabinoxylan, galactomannan and key central metabolic genes were affected in absence of a functional CreA. This study gave a better understanding of the role of CreA during growth on a plant biomass-derived substrate. To conclude, in this chapter we show that CreA plays a major role in transcriptional regulation of utilization of alternative carbon sources and central carbon metabolism during growth of *A. nidulans* on a crude substrate reflecting a condition that is close to the natural environment of this fungus. This will not only increase our knowledge on the function of CreA in filamentous fungi under natural conditions, but this information will be useful for biotechnology for understanding fungal transcriptional regulation on an agricultural substrate.

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Supplementary material

Table S1 | Expression of CAZymes involved in the degradation of plant biomass in *Aspergillus nidulans.* **Available upon request from the author.**

Table S2 | Expression of known gene involved in sugar metabolism in *Aspergillus nidulans.* **Available upon request from the author.**

Figure S1 | Representation of sugar catabolic pathways, including expression profiles of the genes involved in the pathways. (A) D-galacturonic acid pathway; (B) Oxido-reductive (left) and Leloir (right) D-galactose pathway. Gene expression values are presented under the genes and indicated by a color gradient. A decrease in expression is indicated by yellow squares and increased expression is indicated by a dark blue square. Up-regulated genes in *creAΔ4* are in red and down-regulated genes are in green. **Available upon request from the author.**

Chapter 8

Summary and General Discussion

General Discussion

This PhD research was focused on carbon catabolism during growth on plant biomass in *Aspergillus niger*. This project deepens our knowledge on the metabolic pathways related to monosaccharides derived from plant cell wall polysaccharides, and their regulation. We also looked at the fungal enzymes required for hydrolysis of the polysaccharides. This PhD thesis improved the understanding of sugar utilization by fungi and provides leads for fungal metabolic engineering for novel or improved use of crude plant substrates in industrial applications.

Alternative pathways for carbon utilization and the regulation of plant polysaccharide degradation and utilization are discussed separately below. The discussion on alternative metabolic pathways focuses on hexose and pentose catabolism, and the relative contribution of several metabolic pathways to growth on plant biomass. The discussion on the regulation of plant polysaccharide degradation mainly focuses on the major transcription factors (TFs) involved in the degradation of plant biomass based polysaccharides, as well as their induction mechanisms.

Alternative pathways for carbon utilization

Aspergillus uses a variety of catabolic pathways to efficiently convert all the monomeric components of plant biomass [1, 2]. There are several metabolic pathways involved in the catabolism of hexose sugars. The predominant one is glycolysis, which starts with the conversion of D-glucose to D-glucose-6-P by glucokinase and hexokinase. D-fructose is phosphorylated by hexokinase to D-fructose-6P and also enters glycolysis. Other hexoses, like D-galactose, D-mannose and L-rhamnose, are converted by catabolic pathways to substrates that can enter glycolysis. The pentoses D-xylose and L-arabinose enter the pentose catabolic pathway (PCP) resulting in D-xylulose-5P that enters the pentose phosphate pathway (PPP), where it is converted to enter glycolysis. Depending on the substrate and organism, only one metabolic pathway or a combination of them may be available.

Alternative pathways for carbon utilization have been shown to exist in fungi, yeasts and bacteria. While the Leloir pathway is the common pathway for D-galactose catabolism in most organisms [3], a second pathway for D-galactose catabolism (the oxido-reductive pathway) was previously found in *Aspergillus nidulans* [4] and *A. niger* [5]. The pathways for D-galactose, D-glucose/D-fructose and pentose catabolism are discussed below separately. The discussion on the D-galactose catabolism focuses on the comparison between the different metabolic pathways and their contribution for the growth of *A. niger*. The discussion on the other pathways focuses on the interaction between several sugar metabolic pathways and their impact on the growth on crude substrates.

D-galactose catabolism

Two pathways for D-galactose catabolism have been described, the Leloir pathway [6] and the alternative oxido-reductive pathway [5]. The metabolism of D-galactose via the Leloir pathway is a process that has been conserved from *Escherichia coli* through humans [3, 6]. In the Leloir pathway in *A. niger* D-galactose is converted to D-galactose-1P by galactokinase (GalE), while in the alternative oxido-reductive pathway it is converted to D-galactitol, which is then further converted to L-xylo-3-hexulose by galactitol dehydrogenase (LadB). In recent research, we aimed to determine the relative contribution of both galactose catabolic pathways during growth on plant biomass.

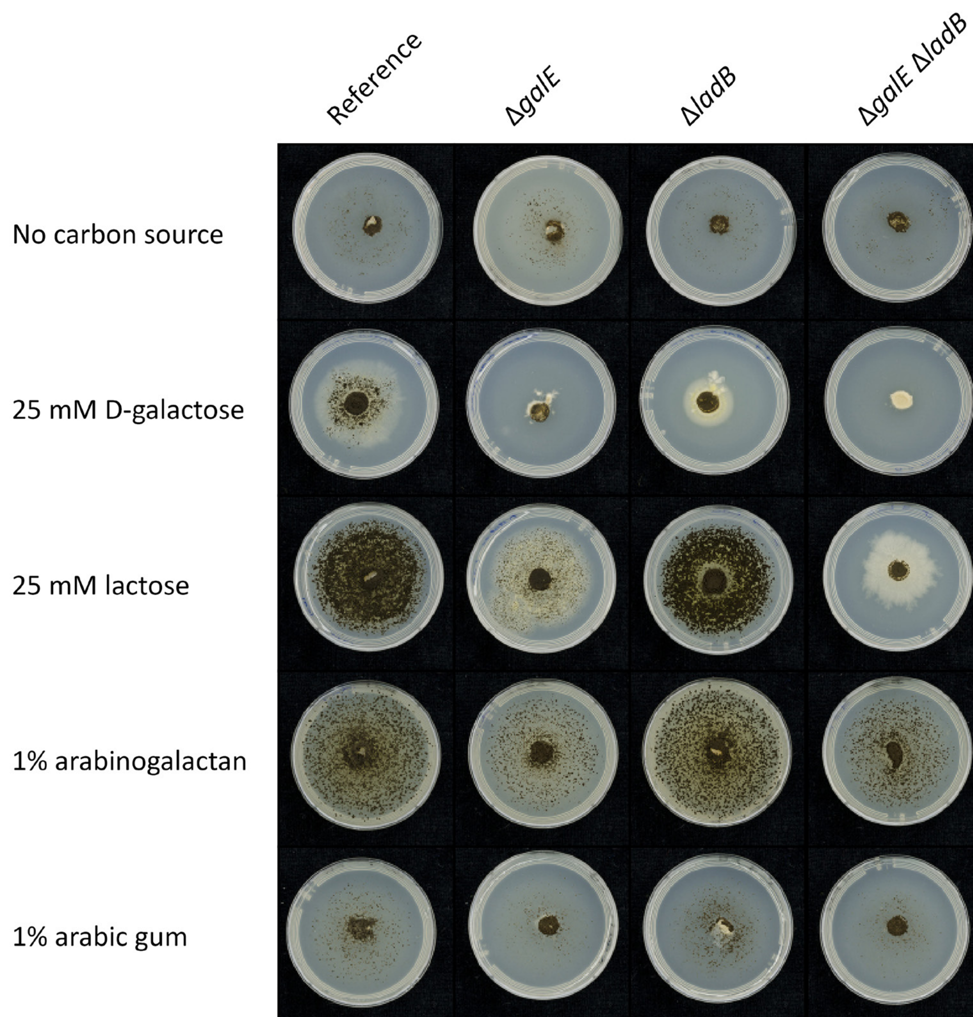

Figure 1 | Growth of the reference, Δ*galE*, Δ*ladB* and Δ*galE* Δ*ladB* *A. niger* strains on no carbon source, D-galactose, lactose, arabinogalactan and arabic gum. All the strains were grown on MM with addition of a carbon source at 30 °C during 7 days.

This study is still in progress, but preliminary results are discussed here due to their relevance for the topic of this thesis.
Although *A. niger* has both catabolic pathways mentioned above, it is not able to grow from spores when D-galactose is the sole carbon source, due to non-functional D-galactose transport in the conidiospores [7, 8]. Single Δ*galE* (NRRL3_06978), Δ*ladB* (NRRL3_07283) and double Δ*galE* Δ*ladB* deletion mutants were made in *A. niger* CBS 141257. Growth profile from mycelium of all the deletion mutants and the reference strain was performed on several carbon sources. Deletion of *galE*, *ladB* or both genes did not affect growth on D-glucose, D-fructose, D-xylose and L-arabinose (data not shown).
Growth of Δ*galE* was reduced on D-galactose, lactose and arabinogalactan compared to the reference, while growth of Δ*ladB* was only reduced on D-galactose compared to the reference (Fig.1). The growth of Δ*galE*Δ*ladB* was abolished on D-galactose and strongly reduced on lactose compared to the reference (Fig.1). As the deletion of *galE* appears to have a stronger effect on D-galactose, lactose and arabinogalactan, this suggests that the Leloir pathway might be the main pathway for D-galactose catabolism in *A. niger*. These preliminary results are not in accordance with a previous study that suggested that the oxido-reductive pathway is the main pathway involved in the D-galactose catabolism in *A. niger* [5]. Transcriptomic analysis may give more insight on the relative contribution of both pathways in the D-galactose catabolism.

Both pentose catabolism and the oxido-reductive galactose pathway involve several dehydrogenases and reductases. *A. niger* uses different metabolic enzymes for these pathways, e.g. L-arabitol dehydrogenase (LadA) for L-arabinose and (LadB) for D-galactose. In contrast, in *Trichoderma reesei* aldose reductase (Xyl1) and L-arabinitol-4-dehydrogenase (Lad1) are involved in both D-galactose and L-arabinose catabolism [9]. To evaluate the effect of the D-galactose catabolic mutants on other pathways, triple mutants were made that combined these mutations with a mutation in a gene involved in the PCP (Δ*galE* Δ*ladB xkiA1*) [10] and glycolysis (Δ*galE* Δ*ladB* Δ*hxkA*). As mentioned above, in *T. reesei* there are links between pentose and D-galactose catabolism. Direct interaction between catabolic pathways has not previously been examined in *A. niger*.
Growth of Δ*galE* Δ*ladB* Δ*hxkA* on lactose and arabinogalactan was even more reduced compared to Δ*galE* Δ*ladB* (Fig.1 and 2A). HxkA appears to play a role in lactose catabolism and arabinogalactan degradation. Growth of the single and double deletion mutants (Δ*galE* and Δ*ladB*) was not affected on GG, SBP and WB (data not shown), but growth of Δ*galE* Δ*ladB* Δ*hxkA* was strongly reduced on these substrates compared to the reference (Fig.2B), most likely due to the important role of *hxkA* in phosphorylating the hexoses that are the main component of these substrates.

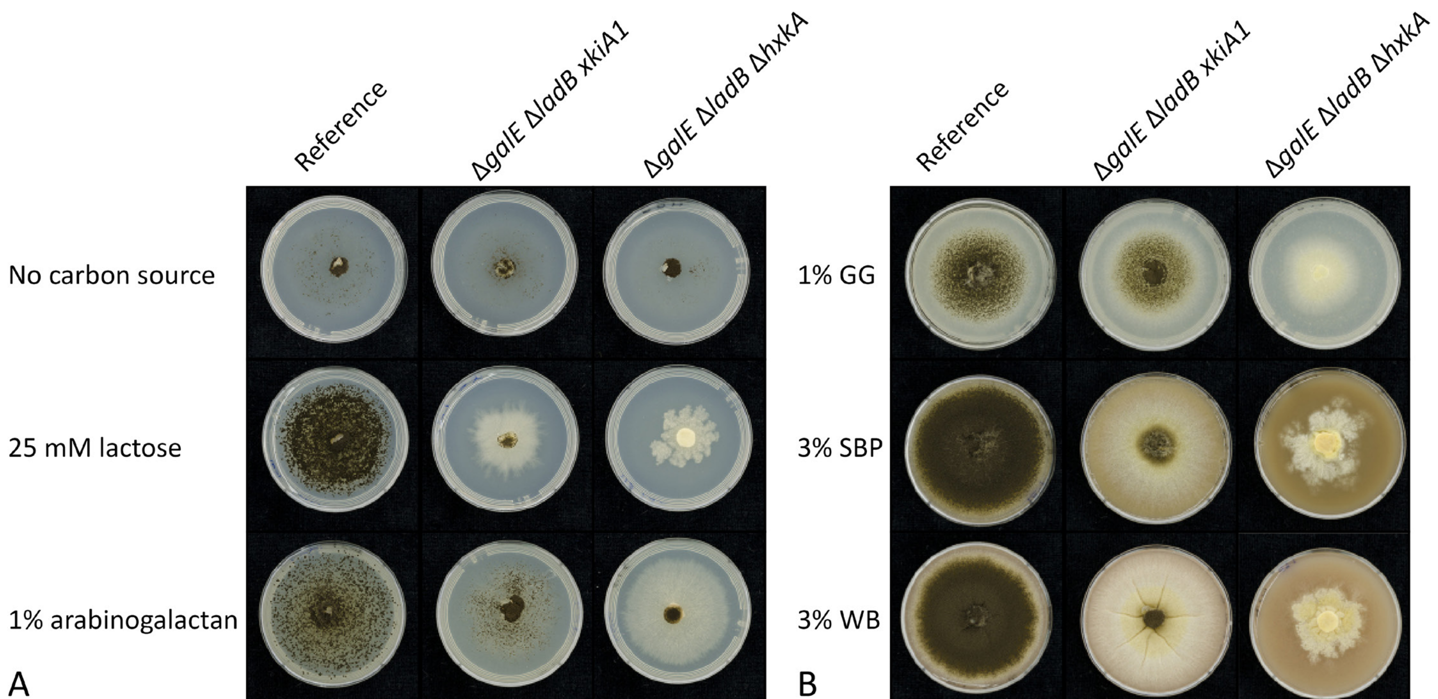

Figure 2 | Growth of the reference, Δ*galE* Δ*ladB xkiA1* and Δ*galE* Δ*ladB* Δ*hxkA* *A. niger* strains on no carbon source, lactose, arabinogalactan, guar gum (GG), sugar beet pulp (SBP) and wheat bran (WB). All the strains were grown on MM with addition of a carbon source at 30 °C during 7 days for the polysaccharides (A) and 3 days for the crude substrates (B).

Growth of *xkiA1* and Δ*galE* Δ*ladB xkiA1* was reduced on GG, SBP and WB (Fig. 2B and data not shown). The effect of the *xkiA1* mutation can be easily explained by the presence of pentoses in SBP and WB. For GG this was less obvious, but analysis of the Sigma GG that was used revealed the presence of monomeric L-arabinose in this stock, which would explain the effect in the *xkiA1* mutant. The results showed that *hxkA* deletion has a stronger effect than *xkiA1* mutation on the growth on different polymeric and crude substrates. Interestingly, HxkA appears to influence growth of *A. niger* in a *galE ladB* negative strain, which could suggest that *A. niger hxkA* may be able to phosphorylate D-galactose to some extent.

The fact that the phenotypes on average were reduction but not absence of growth, suggests that also other pathways or enzymes may play a role in the conversion of several sugars. This then also raises a question about the validation of supposedly known metabolic pathways. To get a better view on the relevance of the pathways, better validation of the pathway enzymes is needed. Different validations can be done: biochemical characterization of the enzymes *in vitro*, functional characterization of the enzymes *in vivo* (e.g. by gene deletion), and demonstration that the genes are expressed under relevant condition. Two of these validations are minimally necessary to confirm the function of a metabolic gene. For instance, in *A. niger* a third pathway for D-galactose catabolism has been described [11], but so far none of the genes or enzymes responsible for the conversions in this pathway have been described.

A. niger is widely used in industry and numerous studies have been devoted to its biology. However, the catabolism of D-galactose remains obscure, even though this hexose is a major component of hemicellulose and pectin. Preliminary data (see above) suggests that both the Leloir pathway and the alternative oxido-reductive pathway are required for D-galactose catabolism in *A. niger*, with a bigger influence of the Leloir pathway on growth on lactose and arabinogalactan. The growth of Δ*ladB* on lactose and arabinogalactan showed that *A. niger* is able to use the Leloir pathway to compensate for the loss of the alternative oxido-reductive pathway. The presence of alternative pathways for the Leloir pathway have also been reported in bacteria and other fungi, but not in yeast [6, 12, 13]. In bacteria, the D-tagatose 6-phosphate pathway [14, 15] and an oxidative pathway via 2-keto-3-deoxy-6-phosphogalactonate have been reported [16]. A deeper understanding of this variation in D-galactose catabolism and the relative contribution of the pathways in different species will require further investigations.

Alternative pathways to convert D-glucose and D-fructose

Chapter 6 demonstrated that in an *A. nidulans* hexokinase glucokinase double mutant, D-glucose and D-fructose are converted through alternative metabolic pathways during growth on wheat bran. In addition, the *creA*Δ*4* mutant in combination with a hexokinase glucokinase deficiency results in an increased expression of PCP and PPP genes (Chapter 6).

This indicates that *A. nidulans* is able to bypass metabolic mutations by using metabolic pathways for other sugars present in plant biomass and suggests that other metabolic pathways or enzymes are involved in the conversion of hexoses and pentoses. The results also indicated that in *A. nidulans*, PPP and PCP can only partially compensate for the loss of *hxkA*, confirming that glycolysis is indeed the major metabolic pathway involved in the sugar catabolism in this fungus during growth on a crude substrate.

The data presented in this thesis suggest that sugar catabolic pathways of *Aspergillus* are highly interconnected and alternative pathways for the main metabolic pathway may exist. While this thesis provides a first view on the contribution of different carbon catabolic pathways to growth of fungi on complex substrates, such as plant biomass, much still remains to be elucidated.

Regulation of plant polysaccharide degradation and conversion

A. niger regulates production of plant polysaccharide degrading enzymes and sugar catabolic enzymes at the transcriptional level. For a fine-tuned response, several transcription factors (TFs) are involved, each controlling a subset of enzymes. To degrade complex components such as plant cell wall polysaccharides, several inducers may be required to adequately respond to the variation in composition and structure of the substrates.

Inducers for pectinolytic gene expression in *A. niger*

The induction of the expression of pectinolytic genes in *A. niger* is complex: some of them are constitutively expressed, while others are specifically induced [17, 18]. The main component of pectin is D-galacturonic-acid (GA), followed by L-rhamnose. Three transcriptional activators (GaaR, RhaR and AraR) and two transcriptional repressors (GaaX, CreA) have been linked to pectin degradation in *A. niger* [17, 19-22]. CreA is discussed in a separate section below.

GaaR is a transcription activator involved in GA utilization and regulates most of the pectinolytic genes [19], while RhaR is a TF activating the expressing of genes involved in L-rhamnose catabolism and pectinolytic genes involved in the degradation of rhamnogalacturonan I (RG-I) [22-24]. The pathways for two major components of pectin, GA and L-rhamnose are in part catalyzed by similar types of enzymes (Fig. 3). In Chapter 3, comparative analysis of GA catabolic pathway deletion mutants (Δ*gaaA,* Δ*gaaB,* Δ*gaaC and* Δ*gaaD*) indicated that 2-keto-3-deoxy-L-galactonate acts as the inducer of the GA-responsive genes, mediated through GaaR (Fig.3). RNA-seq analysis of Δ*gaaC* revealed significant up-regulation of several genes involved in pectin degradation and GA utilization, compared to the other GA catabolic pathway deletion mutants. The same strategy has been used for the L-rhamnose catabolic pathway in Chapter 2.

However, a similar up-regulation was not observed for any of the L-rhamnose catabolic pathway deletion mutants (Δ*lraA,* Δ*lraB* and Δ*lraC*), suggesting that the inducer may be the product from LraC, 2-keto-3-deoxy-L-rhamnonate. However, we were so far not able to identify the gene encoding LraD (Fig. 3), and can therefore not confirm that this compound is the inducer of RhaR.

Alternatively, the pathway in *A. niger* may differ from *Scheffersomyces stipitis* in that it converts 2-keto-3-deoxy-L-rhamnonate to a different product. A possible candidate for this conversion could be a putative alcohol dehydrogenase that contains the same PFAM domains as L-KDR-4-dehydrogenase (KDRDH) from *Sulfobacillus thermosulfidooxidans* [25], indicated in Fig. 3 as LraE. Single (Δ*lraE*) and double deletion (Δ*lraD* Δ*lraE*) mutants may provide more insight on the identity of the RhaR inducer in *A. niger*.

The degradation of plant biomass is more complex than that of pure polysaccharides and requires the synergistic action of several TFs, which respond to different inducers. A recent study [26] revealed that GaaR, AraR and RhaR all contribute to the regulation of pectin degradation in *A. niger* grown on sugar beet pectin (SBP). Chapter 2 and 3 clearly showed that from complex polysaccharides, such as pectin, multiple inducers can be released simultaneously.

Other inducers involved in plant biomass degradation

In *A. niger*, co-regulation of xylanolytic and cellulolytic genes via the inducer D-xylose has been reported [27-29]. It has also been shown that L-arabitol is the inducer of the arabinanolytic and xylanolytic regulatory system [30, 31]. In addition, it has been demonstrated that D-xylose induction is concentration-dependent: at very low concentrations, it acts as an inducer for xylanases, while at higher concentrations as a repressor through CreA, repressing the expression of several xylanolytic genes [32, 33]. The question for future experiments would be, to which extend D-xylose can be called an inducer? Depending on the composition of the substrate (crude plant biomass or simple polysaccharide), the set-up of the experiment (growth conditions, incubation time), the induction of the (hemi) cellulolytic system by D-xylose might differ.

So far, it has not been demonstrated that D-xylose itself is the real inducer of XlnR. In Chapter 2 and 3, single metabolic mutants of all the pathway genes were used to identify the true inducer of GaaR and RhaR. A similar strategy should be used for the PCP to confirm that D-xylose is the true inducer of XlnR. Also, differences in the xylanolytic and arabinanolytic regulatory systems have been observed between *A. niger* and *A. nidulans* [31]. It has been demonstrated that the role of the L-arabinose responsive regulator (AraR) in the PCP differs between these two Aspergilli, while this is not the case for the (hemi-)cellulolytic regulator (XlnR) [31]. Comparative analysis showed differences between growth in the *A. nidulans* and *A. niger* Δ*araR* and Δ*araR*Δ*xlnR* strains.

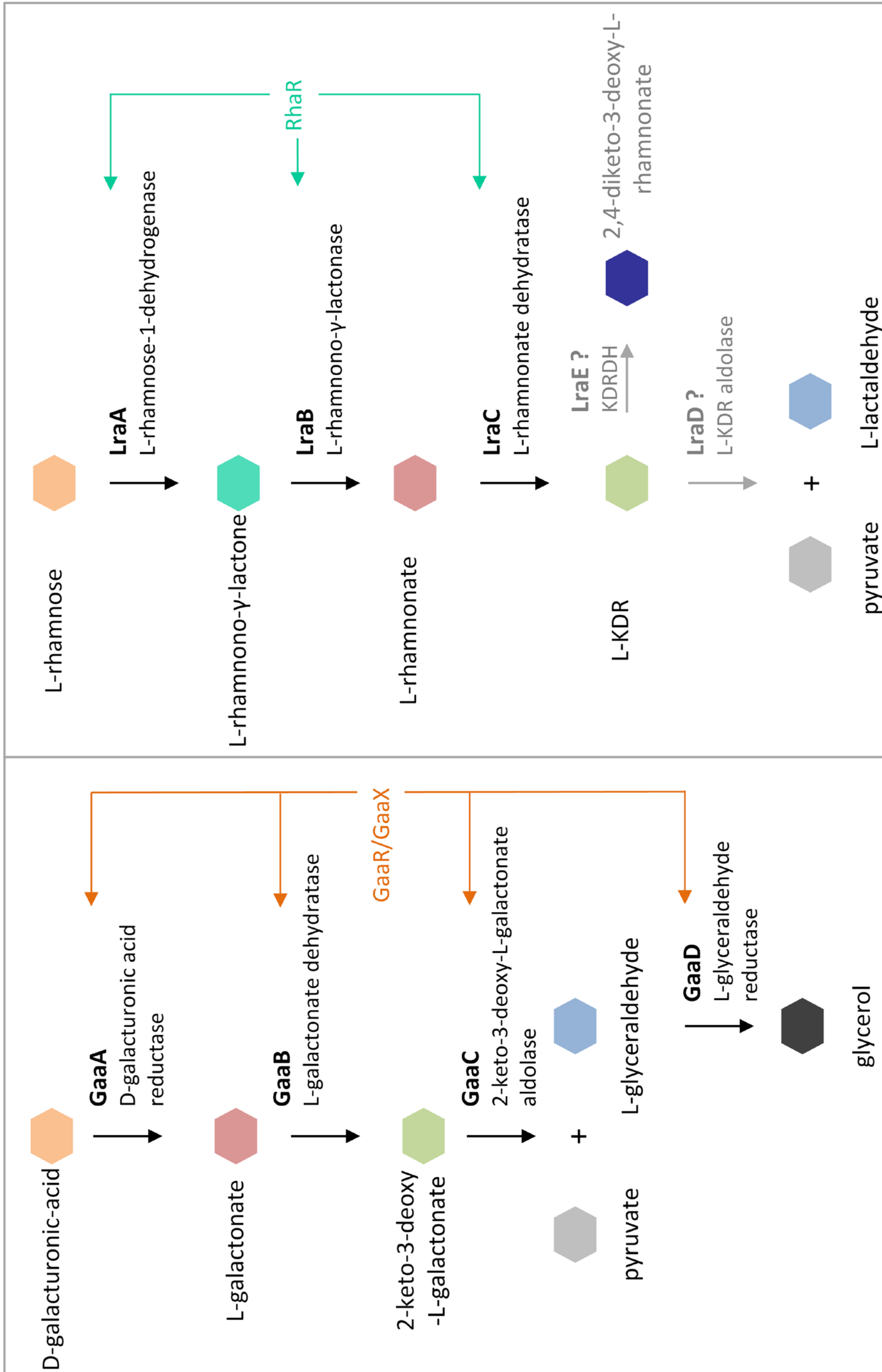

Figure 3 | D-galacturonic-acid and L-rhamnose metabolic pathways in *A. niger.*

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A. nidulans Δ*araR* was impaired on L-arabinose, but only a small growth reduction was observed on L-arabitol, while the *A. niger* Δ*araR* showed only a small growth reduction on L-arabinose, but its growth on L-arabitol was strongly reduced. Growth of the *A. nidulans* double mutant was only slightly affected on L-arabinose, whereas the growth was absent on this carbon source in the *A. niger* double mutant. Unlike in *A. niger*, the absence of AraR and/or XlnR in *A. nidulans* did not affect the growth on xylitol. It has been suggested that an additional regulatory system or an alternative pathway for L-arabinose catabolism might explained the results [31]. XlnR function also varies between these species with respect to its target genes encoding extracellular enzymes (see below). This may indicate that the use of pentoses by fungi may have significant variations in the PCP and its regulatory mechanism. Therefore, we hypothesize that additional/different inducing molecule for the PCP might be used depending on the *Aspergillus* species.

Starch is a common component of plant biomass that can be degraded by most fungi using amylolytic enzymes. The production of these enzymes is induced by starch and maltose in Aspergilli. These substrates are converted by α-glucosidases to the physiological inducer isomaltose in *A. nidulans* [34-36]. Isomaltose appears to be an effective molecule to cause nuclear localization of the amylolytic regulator (AmyR), within a short period and at very low concentrations [37, 38]. In *A. nidulans*, D-glucose was also able to trigger the nuclear localization of AmyR [38]. D-glucose is a much weaker inducer than isomaltose but has the ability to induce α-amylase production [38]. However, this activity would generally be masked by the effect of the carbon catabolite repressor (CreA) at high glucose concentrations. Significant induction of α-amylases by D-glucose was also observed in a creA-defective *A. nidulans* strain [38]. In *A. niger* it has been showed that AmyR regulates genes encoding α- and β-glucosidases, α- and β-galactosidases, α-amylases and glucoamylases [39]. In addition, D-glucose or a metabolic product might be the inducer of AmyR in *A. niger*, and not maltose [39]. Chapter 7 describes that in the *A. nidulans creAΔ4* mutant *amyR* was significantly up-regulated compared to the reference, as well as two amylases (*amyB*, *amyG*), two α-glucosidases (*agdF* and *agdE*) and one glucoamylase (*glaB*).

XlnR is the major TF involved in the plant biomass degradation and colonization in *A. niger*

The xylanolytic/cellulolytic regulator XlnR is present in nearly all filamentous ascomycetes [21, 40], and plays a key role in plant biomass utilization. This regulator has been well characterized in several fungi, such as *A. niger* [27], *T. reesei* [41], *Fusarium graminearum* [42] and *Neurospora crassa* [43]. However, studies of this regulator suggest that the target genes differ between species [44]. The role of XlnR in the production of cellulolytic and xylanolytic enzymes has been extensively studied during growth on both simple and complex sugars. However, the ability of *A. niger* to colonize crude plant biomass, has never been addressed.

In Chapter 5, we analyzed the colonization of unprocessed wheat bran (WB) by *A. niger* Δ*araR*, Δ*xlnR*, Δ*araR* Δ*xlnR* and reference. After 40h of growth on WB, Δ*xlnR* and Δ*araR* Δ*xlnR* could not colonize the smooth surface of the WB. Absence/reduction of several cellulolytic, and arabinoxylanolytic enzymes in the Δ*xlnR* single and double mutant compared to the reference was also observed. These results confirmed that XlnR is the main regulator for the colonization and production of complex substrates-degrading enzymes in *A. niger*, while AraR has a much lower influence. Other regulators such as ClrA/B cannot compensate for the loss of this regulator (Chapter 4 and 5). The dominant influence of XlnR on the production of several enzymes related to plant biomass degradation was also suggested in a previous study in which *A. niger* was grown on steam-exploded sugarcane bagasse (SEB) [45].
In Chapter 4, we evaluated the transcriptional responses of *A. niger* Δ*xlnR,* grown in corn stover (CS) and soybean hulls (SBH). Our results showed that the effect on CAZy gene expression changes over time and depends on the composition of the crude substrates. At a later time point, on SBH, a high number of enzymes acting on pectin were up-regulated in both mutants compared to CS. Interestingly, *rhaR* was up-regulated in SBH in Δ*xlnR*, as were the L-rhamnose catabolic genes. This result indicates that *A. niger* uses RhaR to (partially) compensate for the loss of XlnR on SBH.
Recently, it has been shown that expression of XlnR-dependent targets was up-regulated on sugar beet pectin (SBP) in strains in which *gaaR* was deleted [26]. Two TFs, *clrA* and *clrB*, involved in cellulose degradation were also up-regulated. This might be due to the increased expression levels of *xlnR*, since XlnR was reported to regulate expression of *clrA* and *clrB* in *A. niger* grown on lignocellulose [46]. These results suggest that the TFs GaaR and XlnR might have an antagonistic effect in *A. niger*, similarly to what has been described for XlnR and AraR [30].
The combined results from these studies (Chapter 4 and 5) confirmed that XlnR is the major regulator involved in the colonization and degradation of plant biomass in *A. niger* and that other cellulose regulators cannot take over the role of XlnR. However, on crude substrates rich in pectin such as SBH or SBP we have observed a compensation effect by other regulators. In the absence of XlnR, a pectinolytic regulator (e.g. GaaR or RhaR) will turn on different enzymes that enable the fungus to use different components of the substrate. Such a regulatory system by which metabolic pathway genes are affected by multiple TF especially benefits fungi like *A. niger* that are able to grow on a broad range of natural substrates. Also, the common presence of D-glucose, D-xylose, D-galacturonic-acid and L-rhamnose in natural polymers likely stimulates an interactive regulatory network.

CreA effects in plant biomass conversion

Carbon catabolite repression (CCR) is a common process that selects the energetically most favorable carbon source in nature. CCR represses the utilization of less favorable carbon sources when a better source (e.g. glucose) is available.

Glucose is the preferential monosaccharide (when used as a single carbon source), because it is rapidly metabolized to generate energy for growth. In *A nidulans* and *A. niger*, CCR is mediated by the TF CreA.
This TF is the only one related to plant biomass degradation known so far that is conserved throughout the fungal kingdom, suggesting that the CCR mechanism is conserved in fungi [40]. Even though D-glucose is the most repressing carbon source, CCR in filamentous fungi is also activated by high (mM range) concentrations of other monosaccharides and is mediated through nuclear localization of CreA [47]. In *A. niger*, comparison of a feruloyl esterase (*faeB*) transcription profiles in a wild-type and a *creA* derepressed mutant showed that D-glucose, D-xylose, L-arabinose, sorbitol, D-mannose and D-fructose can induce CCR [48], while to a lesser extent D-galacturonic acid and D-galactose can also trigger CCR. The least repressing carbon sources in this study were L-rhamnose and D-glucuronic acid.
Chapter 7 showed that the *creAΔ4* mutant affects the metabolism of D-xylose and L-arabinose, whereas the metabolism of D-galacturonic acid and L-rhamnose was not affected. Since XlnR has been reported to be under control of CCR in both *A. nidulans* [49] and *A. niger* [32, 33], this could explain the larger effect of CreA on pentose catabolism, as XlnR is one of the major regulators of this pathway.
In Chapter 4, we showed the effect of an *xlnR* deletion on CreA-function, as *creA* was down-regulated at all the time points tested in SBH in Δ*xlnR*. However, in CS, *creA* was only down-regulated at an early time point and up-regulated at later time points in Δ*xlnR*, indicating that the composition of the plant biomass substrate strongly affects the balance between the regulators. The combined results from these studies (Chapter 4 and 7), suggests that XlnR has an indirect effect on CreA. The results also showed that it has a key role in carbon catabolism and the expression of genes involved in complex carbon-source utilization in *Aspergillus*.

All these data suggest the presence of a complex network between inducers and regulatory systems involved in plant biomass utilization in Aspergilli. While the influence of the regulators on the catabolic pathways is easy to understand on pure mono- and polysaccharides, with often very clear phenotype, this is much less clear on crude substrates. The results in my thesis showed that the presence of multiple monosaccharides in the crude substrates result in only partial phenotypes of the regulator mutants, due to compensation by other regulators and pathways. To fully understand the contribution of the different pathways to the conversion of crude substrates, individual and combined deletion strains of crucial genes in all the pathways need to be made and their phenotype compared during growth on crude substrates.

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Nederlandse samenvatting

Résumé Français

Curriculum vitae

Acknowledgements

Plantenbiomassa is de voornaamste hernieuwbare grondstof op aarde en een belangrijk startmateriaal voor verschillende industriële sectoren. De productie van alternatieve brandstoffen, zoals bioethanol, en biochemicaliën door middel van enzymen die plantenbiomassa afbreken is een sterk groeiende industriële sector. Het substraat voor deze processen bestaat uit plantenmateriaal dat afkomstig is van gewassen die speciaal voor dit doel gekweekt zijn en van landbouwafval. Planten polysachariden kunnen door schimmel enzymen omgezet worden in fermenteerbare suikers. Deze suikers worden dan via fermentatie omgezet in ethanol en ander producten, waarvoor voornamelijk de gist *Saccharomyces cerevisiae* gebruikt wordt. Species van de schimmel *Aspergillus* horen bij de voorkeursorganismen met betrekking tot enzymproductie voor de afbraak van plantenmateriaal omdat zij hoge eiwit niveaus uitscheiden en een breed scala aan enzymen produceren die planten polysachariden kunnen afbreken. In de natuur breekt *Aspergillus* deze polysachariden af om monomere suikers te krijgen die als koolstofbron gebruikt kunnen worden. *Aspergillus* gebruikt een variatie aan katabole routes om alle monomere bestanddelen van plantenbiomassa om te zetten.

In mijn proefschrift heb ik gefocust op verschillende katabole routes van *Aspergillus niger* en *Aspergillus nidulans* die betrokken zijn bij het omzetten van de belangrijkste monomeren (D-glucose, D-xylose, L-arabinose, L-rhamnose en D-galacturonzuur) die in planten polysachariden voorkomen, en op de regulatie van deze routes.

In Hoofdstuk 2 hebben we de functie van de eerste drie genen van de L-rhamnose route bevestigd door middel van gen deletie. Daarnaast hebben we de identiteit van de inducer van de regulator (RhaR) van de route onderzocht door expressie analyse in de deletie mutanten. Hieruit bleek dat de inducer een intermediaire metaboliet van de route is na LraC, waarschijnlijk 2-keto-3-L-deoxyrhamnonaat. Aangezien we nog niet in staat zijn geweest het gen voor de volgende stap (LraD) te identificeren, konden we dit nog niet bevestigen. Dezelfde strategie is ook gebruikt voor de D-galacturonzuur katabole route in Hoofdstuk 3. Mutanten in deze route zijn gemaakt en gebruikt voor groeiprofielen en expressie analyse. Dit toonde aan dat 2-keto-3-deoxy-L-galactonaat de inducer is van de genen die nodig zijn voor het vrijmaken en omzetten van D-galacturonzuur.

D-Xylose en L-arabinose zijn de meest voorkomende monosacchariden na D-glucose in bijna alle plant-gebaseerde materialen. In Hoofdstuk 4 hebben we het effect van de (hemi-) cellulolytische regulator XlnR en xylulokinase (XkiA) mutanten van *A. niger* bestudeert gedurende groei op twee pentose-rijke substraten, mais stro en sojaboon pellen, door middel van expressie analyse. Op latere tijdstippen werden significante verschillen gevonden in de expressie profielen van de mutant als de twee substraten vergeleken werden. Deze studie laat zien hoe complex het plantenbiomassa afbrekingsproces is in schimmels, aangezien mutanten met redelijk eenvoudige fenotypes op simpele suikers een veel minder eenvoudig fenotype en expressie profiel hadden op ruwe plantenbiomassa.

XlnR en de arabinanolytische regulator AraR controleren de productie van enzymen betrokken bij de afbraak van arabinoxylan en katabolisme van L-arabinose en D-xylose in *A. niger*. In Hoofdstuk 5 hebben we kolonisatie en afbraak van tarwe zemelen door de *A. niger* referentie stam en *araR/xlnR* mutanten door middel van hoge-resolutie microscopie en exo-proteomics. Hiermee konden wij de kolonisatie en afbraak efficiëntie van de mutanten verbinden met de afwezigheid van subsets van bepaalde enzymen.

In de biobased industrie worden schimmels gebruikt om lignocellulolytische enzymen te produceren die landbouw afval kunnen afbreken. In Hoofdstuk 6 hebben wij bestudeert of het mogelijk is om een *A. nidulans* stam te maken die de hexosen van plantenbiomassa wel vrij maakt, maar niet metaboliseert. Hiervoor werden mutanten in de glycolyse gemaakt, die geen hexokinase en glukokinase meer hadden, gecombineert met een mutatie in *creA*.

Suiker analyse toonde aan dat hexosen niet ophoopten tijden de groei van de mutanten op tarwe zemelen, wat suggereert dat glucose metabolisme in deze mutanten via een alternatieve route gebeurt. De combinatie van de *creAΔ4* mutatie met het voorkomen van de initiële fosforylering in de glycolyse zorgde voor een verhoging van de expressie van de genen van de pentose katabole route en de pentose fosfaat route. Dit laat zien dat een verlaagde mogelijkheid om hexosen te gebruiken als koolstofbron een verschuiving creëerde naar de pentose fractie van tarwe zemelen als de belangrijkste koolstofbron om groei te ondersteunen.

Koolstof kataboliet repressie (KKR) is een belangrijk regulatie systeem in de meeste micro-organismen. KKR zorgt ervoor dat de meest energetisch voordelige koolstofbron wordt gebruikt en onderdrukt het gebruik van alternatieve koolstofbronnen als een betere aanwezig is. In de filamenteuze schimmel *A. nidulans* is CreA de regulator van het KKR systeem. In Hoofdstuk 7 hebben we de rol van CreA geëvalueerd tijdens groei op tarwe zemelen, dat slechts lage concentraties vrije monosachariden bevat. Expressie analyse toonde aan dat de expressie van CAZy genen betrokken bij de afbraak van cellulose, arabinoxylan en galactomannan, evenals belangrijke metabole genen veranderde in de *creAΔ4* mutant. Dit laat zien dat CreA al een invloed heeft op de fysiologie van schimmels bij lage concentraties van monosachariden, wat een aanwijzing is voor een belangrijke rol voor deze regulator in de natuurlijke biotopen van schimmels.

Al deze data suggereert de aanwezigheid van een complex netwerk van inducers en regulatie systemen dat betrokken is bij de omzetting van plantenbiomassa door Aspergilli. Terwijl de invloed van de regulatoren op de katabole routes makkelijk te begrijpen is bij groei op zuivere mono- en polysacchariden, is dit een stuk minder duidelijk op ruwe substraten.

La biomasse végétale est le principal matériau renouvelable sur terre. Elle est la matière première utilisée dans de nombreuses industries. Un secteur industriel croissant dans lequel les enzymes dégradant les plantes sont utilisées est la production de carburants alternatifs, tels que le bioéthanol et autres produits biochimiques. Le substrat de ces procédés est la biomasse végétale, soit à partir de cultures spécialement cultivées à cet effet, soit à partir de déchets agricoles. Les polysaccharides végétaux peuvent être transformés en sucres par des enzymes fongiques. Ces sucres peuvent ensuite être fermentés en éthanol, principalement par la levure (*Saccharomyces cerevisiae*). Les espèces d'Aspergillus sont des organismes de choix pour la production d'enzymes servant au prétraitement de la biomasse végétale. En effet, elles possèdent des taux élevés de sécrétion de protéines et produisent une large gamme d'enzymes pour la dégradation des polysaccharides des plantes. En milieu naturel, *Aspergillus* dégrade les polysaccharides en sucres monomères, utilisés ensuite comme source de carbone par le champignon. De surcroit, *Aspergillus* utilise une variété de voies cataboliques pour convertir efficacement tous les monomères de la biomasse végétale.
Durant ma thèse, je me suis intéressée aux principales voies cataboliques du carbone chez les espèces *Aspergillus niger* et *Aspergillus nidulans,* impliquées dans la conversion et la régulation des principaux monomères (D-glucose, D-xylose, L-arabinose, L-rhamnose et acide D-galacturonique) présents dans les polysaccharides des plantes.
Dans le chapitre 2, nous avons confirmé la fonction des trois premiers gènes putatifs impliqués dans la dégradation du L-rhamnose chez *A. niger* en utilisant des knock-out mutant. De plus, nous avons exploré l'identité de l'inducteur du rhamnose régulateur (RhaR) à travers l'analyse de l'expression de ces mutants. Ce travail a montré que l'inducteur de RhaR est situé après rhamnonate- L-dehydratase (LraC) et est probablement le 2-kéto-3-L-deoxyrhamnonate. Cependant, nous n'avons pas encore pu identifier le gène codant pour LraD et ne pouvons donc pas confirmer que ce composé est l'inducteur de RhaR. La même stratégie a été utilisée pour la voie catabolique de l'acide D-galacturonique au chapitre 3. Des mutants knock-out de la voie catabolique de l'acide D-galacturonique ont été utilisés, avec lesquels des profils de croissance et une analyse d'expression ont été réalisés. Les résultats obtenus indiquent que le 2-kéto-3-déoxy-L-galactonate est l'inducteur des gènes requis pour l'utilisation de l'acide D-galacturonique.
Le D-xylose et le L-arabinose sont les monosaccharides les plus abondants après le D-glucose dans presque tous les matériaux dérivés de la biomasse végétale. Dans le chapitre 4, nous avons évalué l'effet sur le transcriptome de deux mutants d'*A. niger*, le regulateur (hemi-) cellulolytique (xlnR) et le xylulokinase (xkiA1) lors de la croissance sur deux substrats riches en pentose, à savoir les déchets du maïs (CS) et les coques de soja (SBH). Après 48h de croissance, des différences significatives ont été trouvées dans les profils d'expression des deux mutants pour le substrat CS comparativement au SBH.
Cette étude démontre la grande complexité du processus de dégradation de la biomasse végétale des champignons, en montrant que les mutants avec des phénotypes relativement simples sur mono et polysaccharides purs ont des phénotypes et des transcriptomes beaucoup moins clairs sur la biomasse végétale brute.

Chez *A. niger*, XlnR et le facteur de transcription arabinanolytique (AraR) régulent la production d'enzymes impliquées dans la dégradation de l'arabinoxylane et le catabolisme du L-arabinose et D-xylose. Au chapitre 5, nous avons étudié la colonisation et la dégradation du son de blé par la souche de référence *A. niger* et par les mutants des régulateurs araR / xlnR, à l'aide de la microscopie haute résolution et d'une analyse exo-protéomique a été réalisé. Cette étude nous a permis d'associer la réduction de l'efficacité de colonisation et de dégradation des mutants à l'absence de certains groupes d'enzymes.

Dans les bio-industries, les champignons sont utilisés pour produire des enzymes lignocellulolytiques afin de dégrader la biomasse des déchets agricoles. Dans le chapitre 6, nous avons évalué la possibilité de créer une souche d' *A. nidulans* qui libère mais ne métabolise pas les hexoses de la biomasse végétale. Dans ce but, des mutants métaboliques ont été créés, en utilisant des souches négatives à l'hexokinase (*hxkA*) et à la glucokinase (*glkA*) (impliqués dans la conversion du D-glucose en D-glucose-6-phosphate), combinées à une mutation du carbone catabolique répresseur (*creA*). L'analyse des sucres a révélé que les hexoses ne s'accumulaient pas pendant la croissance des mutants sur le son de blé, ce qui suggère que le métabolisme du glucose est détourné vers d'autres voies cataboliques. La mutation *creAΔ4* associée à la prévention de la phosphorylation initiale dans la glycolyse a entraîné une augmentation de l'expression des gènes impliqués dans la voie catabolique des pentoses. Les résultats obtenus indiquent donc que la capacité réduite à utiliser des hexoses comme source de carbone a créé un décalage vers la fraction pentose du son de blé en tant que principale source de carbone pour soutenir la croissance.

La répression des métabolites du carbone (CCR) est un système de régulation clé dans la plupart des microorganismes. CCR est un processus qui garantit l'utilisation de la source de carbone la plus favorable dans un environnement donné et réprime l'utilisation d'une source de carbone alternative. Dans le champignon filamenteux *A. nidulans*, CCR est médié par le facteur de transcription CreA. Au chapitre 7, nous avons évalué le rôle de CreA pendant la croissance sur un substrat plus naturel, le son de blé, qui ne contient que de faibles niveaux de monosaccharides libres. L'analyse transcriptomique a montré que les niveaux d'expression des gènes CAZy impliqués dans la dégradation de la cellulose, de l'arabinoxylane et du galactomannane ont été affectés par la mutation *creAΔ4*. En conclusion, nos résultats ont montré que CreA influence la physiologie des champignons à de faibles niveaux de monosaccharides libres, ce qui suggère un rôle important de ce régulateur dans les habitats naturels des champignons.

Toutes ces données supportent la présence d'un réseau complexe entre les inducteurs et les systèmes de régulation impliqués dans l'utilisation de la biomasse végétale chez Aspergilli. Bien que l'influence des régulateurs sur les voies cataboliques soit facile à comprendre pour les mono- et polysaccharides purs, avec un phénotype souvent très clair, cela est beaucoup moins clair sur les substrats bruts, montrant la complexité de tel réseaux et leur régulation.

Claire Khosravi was born on May the 25th, 1990 in Nice, France. After high school, she followed her second education at the University of Nice-Sophia Antipolis, France and graduated in 2011 with a Bachelor-level diploma in Life and Health Sciences. In September of the same year, she began her study in Human Nutrition and Health at the University of Bordeaux. As part of her master, she did her first internship at the research Unit Mycology and food safety (MycSA), National Institute for Agricultural Research (INRA), Bordeaux, France, under supervision of Dr. F. Forget-Richard, followed by a second internship in the same group, under supervision of Dr. M. Montibus and Dr. F. Forget-Richard. Claire obtained her MSc diploma in June 2013. In August of the same year, she started her PhD with the Fungal Physiology group, at the Westerdijk Fungal Biodiversity institute, under supervision of Prof. Dr. R.P. de Vries.

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*co-first authors.

Posters and Presentations

Khosravi C, Kun RS, Visser J, Aguilar-Pontes MV, de Vries RP, Battaglia E; the 2nd Symposium on Plant Biomass Conversion by Fungi, 28th-29th August 2017, Utrecht, the Netherlands (poster award)

Khosravi C, Kun RS, Visser J, Aguilar-Pontes MV, de Vries RP, Battaglia E; the 29th Fungal Genetics Conference, 14th-19th March 2017, Pacific Grove, California, USA (poster presentation)

Khosravi C, Kun RS, Visser J, Aguilar-Pontes MV, de Vries RP, Battaglia E; the 14th International Aspergillus Meeting (Asperfest14), 13th-14th March 2017, Pacific Grove, California, USA (poster presentation)

Khosravi C, Kun RS, Visser J, Aguilar-Pontes MV, de Vries RP, Battaglia E; Annual meeting of the section mycology of the KNVM, 25th November 2016, Utrecht, The Netherlands (oral presentation)

Khosravi C, Battaglia E, Aguilar-Pontes MV, Dalhuijsen S, Zhou M, Heyman H, Kim YM, Baker S, de Vries RP; MB3.0 Fall meeting Microbial Biotechnology section of the KNVM, 7th November 2016, Delft, The Netherlands (oral presentation)

Khosravi C, Battaglia E, Aguilar-Pontes MV, Dalhuijsen S, , Zhou M, Heyman H, Kim YM, Baker S, de Vries RP; MB3.0 fall meeting Microbial Biotechnology section of the KNVM, 7th November 2016, Delft, The Netherlands (poster award)

Kowalczyk JE - **Khosravi C**, Purvine S, Dohnalkova A, Chrisler WB, Orr G, Robinson E, Zink E, Wiebenga A, Battaglia E, Benoit I, Baker S, de Vries RP; PYFF6: 6th Conference on Physiology of Yeast and Filamentous Fungi, Lisbon, Portugal, July 11-14 2016 (poster presentation)

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Khosravi C, Battaglia E, Benoit I, de Vries RP the 11th International Aspergillus Meeting (Asperfest 11), 11th-12th March 2014, Seville, Spain (poster presentation)

Khosravi C, Benoit I, de Vries RP; the XI International Fungal Biology Conference, 29th September-3rd October 2013, Karlsruhe, Germany (poster presentation)

Here we are, the last pages of my book! Everything started 4 years ago and it feels like it was yesterday… At the back of my mind, I often wondered: how do I get from here to there, to this book? Now I have more clues to answer that question.
Obtaining a PhD is like swimming across a big lake. I believe, of course, that it is indeed possible to learn to swim, and even to do it well. In fact, I think that mastering certain skills along the way is just as important as swimming across the lake to get to the prize on the other side: your PhD!
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