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Detailed analysis of the D-galactose catabolic pathways in *Aspergillus niger* reveals complexity at both metabolic and regulatory level

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ABSTRACT

The current impetus towards a sustainable bio-based economy has accelerated research to better understand the mechanisms through which filamentous fungi convert plant biomass, a valuable feedstock for biotechnological applications. Several transcription factors have been reported to control the polysaccharide degradation and metabolism of the resulting sugars in fungi. However, little is known about their individual contributions, interactions and crosstalk. D-galactose is a hexose sugar present mainly in hemicellulose and pectin in plant biomass. Here, we study D-galactose conversion by *Aspergillus niger* and describe the involvement of the arabinanolytic and xylanolytic activators AraR and XlnR, in addition to the D-galactose-responsive regulator GalX. Our results deepen the understanding of the complexity of the filamentous fungal regulatory network for plant biomass degradation and sugar catabolism, and facilitate the generation of more efficient plant biomass-degrading strains for biotechnological applications.

1. Introduction

Plant biomass is the most abundant renewable resource in the terrestrial biosphere. Filamentous fungi are able to degrade complex plant cell wall polysaccharides (cellulose, hemicelluloses and pectins) into their monomeric building blocks, offering great potential for an increasing number of biotechnological applications (de Vries and Visser, 2001; de Vries et al., 2020). Plant biomass degradation by fungi is mediated through the production of a broad range of extracellular enzymes, the production of which is controlled by transcription factors (TFs) and linked to the activation of metabolic pathways that allow the utilization of the released monomers as carbon sources (Kowalczyk et al., 2014; Khosravi et al., 2015; Benocci et al., 2017).

D-galactose is a six-carbon monosaccharide present primarily in hemicelluloses, pectins and gums, and often co-occurs with the five-carbon monosaccharides L-arabinose and D-xylose (Kowalczyk et al., 2014). In filamentous fungi, two different pathways have been shown to be involved in D-galactose catabolism: the Leloir pathway (Frey, 1996) and the oxido-reductive catabolic pathway (Fekete et al., 2004) (Fig. 1). However, some enzymes and intermediate compounds involved in these pathways differ among fungal species (Seiboth et al., 2007). The Leloir pathway converts D-galactose into D-glucose-6-phosphate, which subsequently enters glycolysis. The oxido-reductive pathway, in contrast, converts D-galactose into D-fructose-6-phosphate (Fekete et al., 2004; Seiboth et al., 2004; Koivistoinen et al., 2012; Mojzita et al., 2012a), and in some fungal species involves enzymes from the Pentose Catabolic Pathway (PCP) (Pail et al., 2004; Seiboth et al., 2007; Flipphi et al., 2009) (Fig. 1), which is responsible for the catabolism of L-arabinose and D-xylose (Hasper et al., 2000; Hasper et al., 2004; de Groot et al., 2007). Several genes involved in this pathway have been identified in *Aspergillus niger* and have been deleted or the corresponding enzymes have been (partially) characterized (Chroumpi et al., 2021). Analysis of

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deletion strains of various D-galactose oxido-reductive catabolic genes (*ladB*, *xyrA*, *sdhA*, *xhrA*) resulted in reduction of growth on D-galactose and/or galactitol, suggesting their involvement in this pathway (Koivistoinen et al., 2012; Mojzita et al., 2012b; Mojzita et al., 2012a). These phenotypes also suggested a major role for this pathway in growth of *A. niger* on D-galactose.

The co-existence of D-galactose, L-arabinose and D-xylose in nature, along with their common structural features probably stimulated the evolution of an interactive regulatory network in which several TFs co-regulate the expression of the same target genes. Previous studies demonstrated that filamentous fungi are able to respond similarly to the presence of different sugars. For instance, D-galactose and L-arabinose have been reported to be consumed simultaneously in the fungus *Aspergillus nidulans*, and genes involved in D-galactose catabolism were reported to be also induced by the presence of L-arabinose (Németh et al., 2019).

GalX is the main regulator of the D-galactose oxido-reductive pathway in *A. niger*, but does not appear to be directly involved in the control of the Leloir pathway-related genes (Gruben et al., 2012). Regulation of the PCP-related genes is controlled by the transcriptional activators AraR and XlnR (van Peij et al., 1998; Battaglia et al., 2011b; Battaglia et al., 2014). XlnR and AraR control a wide range of target genes encoding (hemi-)cellulose and arabinan degrading enzymes and the enzymes involved in D-xylose and L-arabinose metabolism, respectively (de Groot et al., 2007; Battaglia et al., 2011b) (Fig. 1; PCP).

Carbon catabolite repression (CCR) is a regulatory mechanism in which the presence of easily metabolizable carbon sources (e.g., D-glucose) represses the expression of genes that are involved in the utilization of alternative, less-preferred, carbon sources (Ruijter et al., 1997; Brown et al., 2014). This mechanism ensures the optimal utilization of the fungal energy resources, since an increasing concentration of free and rapidly metabolizable sugars in the environment promote the repression of genes encoding for enzymes involved in the degradation of complex polysaccharides (Kowalczyk et al., 2014; Benocci et al., 2017). The repressor CreA has long been recognized as the key TF mediating the CCR in fungi (Ronne, 1995; Ruijter et al., 1997; Strauss et al., 1999), and a *creA* deletion has been demonstrated to upregulate many genes encoding polysaccharide-degrading enzymes (Peng et al., 2021).

The characterization of genes involved in sugar catabolism and their regulatory mechanisms in filamentous fungi provides a conceptual framework that would allow us to design strategies to improve fungal cell factories, which has direct implications at the biotechnological and industrial level. Although D-galactose catabolism and its regulation have been studied in depth in some filamentous fungi, such as *A. nidulans* (Kowalczyk et al., 2015), several aspects of this in *A. niger* remain unclear. In this study, we investigated the two main D-galactose catabolic pathways in *A. niger* and evaluated their relative contribution to growth on this sugar. In addition, we studied possible interactions between the D-galactose-responsive regulator, GalX, and the arabinanolytic and (hemi-)cellulolytic regulators, AraR and XlnR, in D-galactose catabolism. For this, we generated single and combinatorial deletion mutants of the genes encoding the metabolic enzymes and these three TFs, and studied their phenotype and transcriptomic profile on different carbon sources. Additionally, the contribution of the main carbon catabolite repressor CreA in D-galactose catabolism in *A. niger* was addressed for the first time. For this, we deleted the *creA* gene in all the previous TF mutant sets and studied and compared their phenotypic behavior.

2. Material and methods

2.1. Strains, media and growth conditions

Escherichia coli DH5α was grown in Lysogeny Broth (LB) supplemented with 50 μg/mL ampicillin (Sigma-Aldrich), and was used for plasmid propagation. All *A. niger* strains used and generated in this study were deposited at the culture collection of Westerdijk Fungal Biodiversity Institute under accession numbers listed in Table 1. The fungal strains were grown at 30 °C using Minimal Medium (MM) or Complete Medium (CM) with the appropriate carbon source (de Vries et al., 2004). For solid cultivation, 1.5% (w/v) agar was added in the medium and all agar plates contained 1% D-glucose as carbon source unless otherwise stated. As required, media of auxotrophic strains were supplemented with 1.22 g/L uridine (Sigma-Aldrich).

For growth profiling, MM agar supplemented with 25 mM D-glucose, 25 mM L-arabitol, 25 mM D-xylose, 25 mM xylitol, 25 mM D-galactose, 25 mM galactitol, 25 mM D-sorbitol, 25 mM D-fructose or 25 mM L-arabinose, or a mixture of 25 mM D-galactose and 2 mM L-arabinose was used. All substrates were obtained from Sigma-Aldrich. Spores were harvested from CM agar plates in ACES buffer after five days of growth, and concentration was adjusted using a haemocytometer. Growth profiling plates were inoculated in duplicate with 2 μL spore solution

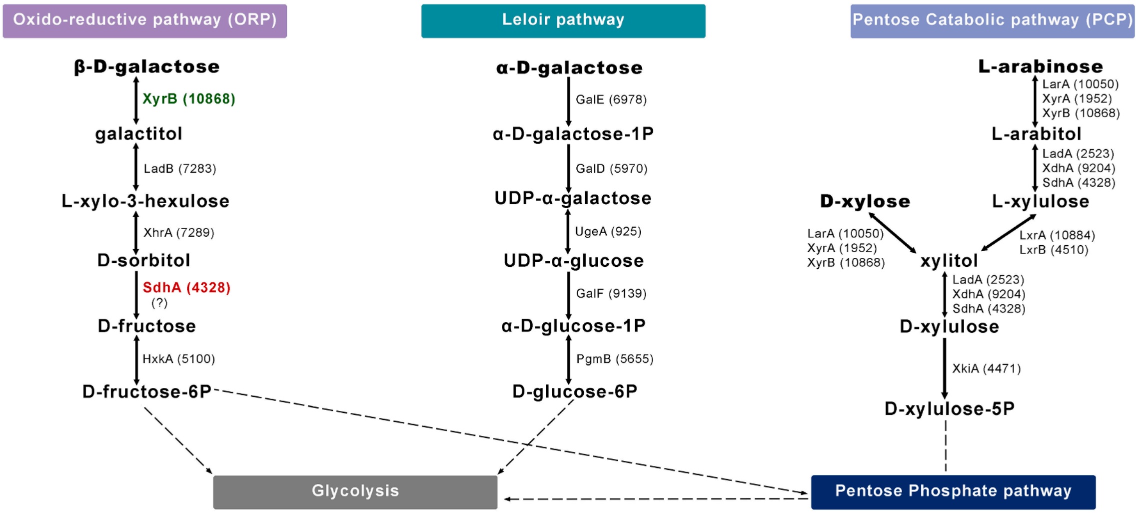


Fig. 1. Oxido-reductive D-galactose pathway (ORP), Leloir pathway and pentose catabolic pathway (PCP) in *A. niger*. LadB = galactitol dehydrogenase, XhrA = L-*xylo*-hexulose reductase, SdhA = D-sorbitol dehydrogenase, HxkA = hexokinase, GalE = galactokinase, GalD = Galactose-1-phosphate uridylyltransferase, UgeA = UDP-galactose 4-epimerase, GalF = UTP-glucose-1-phosphate uridylyltransferase, PgmB = phosphoglucomutase, LarA = L-arabinose reductase, LadA = L-arabitol dehydrogenase, LxrA and LxrB = L-xylulose reductases, SdhA = sorbitol dehydrogenase, XyrA and XyrB = D-xylose reductases, XdhA = xylitol dehydrogenase, XkiA = D-xylulose kinase. XyrB was identified in this study and is indicated in green font.

Table 1
A. niger strains used in this study.

Strains	Gene ID	CBS number	Genotype	Reference
Reference strain (N593 Δ*kusA*)	–	CBS 138852	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$	(Meyer et al., 2007)
Δ*galE*	NRRL3_06978 (*galE*)	CBS 144058	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***galE***$^{-}$	This study
Δ*ladB*	NRRL3_07283 (*ladB*)	CBS 144055	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***ladB***$^{-}$	This study
Δ*galE*Δ*ladB*	–	CBS 145933	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***galE***$^{-}$***,*** ***ladB***$^{-}$	This study
Δ*galX*	NRRL3_07290 (*galX*)	CBS 146900	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***galX***$^{-}$	(Garrigues et al., unpublished)
Δ*xlnR*	NRRL3_04034 (*xlnR*)	CBS 145447	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***xlnR***$^{-}$	(Kun et al., 2021)
Δ*araR*	NRRL3_07564 (*araR*)	CBS 145451	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***araR***$^{-}$	(Kun et al., 2021)
Δ*galX*Δ*xlnR*	–	CBS 147097	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***galX***$^{-}$***,*** ***xlnR***$^{-}$	This study
Δ*araR*Δ*xlnR*	–	CBS 145455	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***xlnR***$^{-}$***,*** ***araR***$^{-}$	(Kun et al., 2021)
Δ*araR*Δ*galX*	–	CBS 147096	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***galX***$^{-}$***,*** ***araR***$^{-}$	This study
Δ*galX*Δ*xlnR*Δ*araR*	–	CBS 147098	*A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***galX***$^{-}$***,*** ***xlnR***$^{-}$***,*** ***araR***$^{-}$	This study

(500 spores per μL) and incubated at 30 °C for up to 13 days. Growth was monitored daily by visual inspection.

Liquid cultures were incubated in an orbital shaker at 250 rpm and 30 °C. For transfer experiments, the pre-cultures containing 250 mL CM with 2% D-fructose in 1 L Erlenmeyer flasks were inoculated with 10^6 spores/ml and incubated for 16 h. Thereafter, the mycelia were harvested by filtration on sterile cheesecloth, washed with MM and ~0.5 g (dry weight) were transferred to 250 mL Erlenmeyer flasks containing 50 mL MM supplemented with 25 mM glycerol, 25 mM D-glucose, 25 mM D-galactose, 5 mM L-arabinose, a mixture of 25 mM D-galactose and 25 mM glycerol, or a mixture of 25 mM D-galactose and 5 mM L-arabinose. All cultures were performed in triplicate. After 2 h of incubation, the mycelia were harvested by vacuum filtration, dried between tissue paper and frozen in liquid nitrogen. All samples were stored at −80 °C until being processed.

2.2. DNA construction, protoplast-mediated transformation, and mutant purification

For the generation of all *A. niger* mutants described in this study, the CRISPR/Cas9 genome editing system was used (Song et al., 2018). The design of the 20 bp protospacers for the gRNAs was performed using Geneious 11.04.4 software (https://www.geneious.com). *A. niger* NRRL3 genome was used as reference (Vesth et al., 2018). The gRNA sequences (Supplementary Table 1) with no predicted off-targets and the highest on-target activity were designed based on the experimentally determined predictive model described by (Doench et al., 2014). The gRNAs were obtained to delete the D-galactose catabolic genes *galE* (gene ID: NRRL3_06978) and *ladB* (gene ID: NRRL3_07283), and the transcription factor-encoding genes *xlnR* (gene ID: NRRL3_04034), *araR* (gene ID: NRRL3_07564) (Kun et al., 2021), and *galX* (gene ID: NRRL3_07290) (Garrigues et al., unpublished).

To construct linear deletion DNA cassettes (also known as rescue templates, RT), we amplified ~500 bp of the upstream and downstream flanking regions of all the candidate genes by PCR using gene specific primers (Supplementary Table 1). We performed PCR amplification using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific), following manufacturer's instructions. Genomic DNA from reference strain (CBS 138852) was used as a template. The upstream reverse and the downstream forward primers were designed to harbor a barcode sequence [actgctaggattcgctatcg]. This sequence was used as the homologous region for the fusion of these two fragments in a subsequent PCR reaction, to generate the linear deletion DNA cassette. The amplified deletion cassettes were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega).

CRISPR/Cas9 plasmid construction, generation of *A. niger* protoplasts, transformation and purification of mutant strains were performed as previously described (Kun et al., 2020). All transformations were carried out using 1 μg of ANEp8-Cas9-*pyrG*-gRNA plasmid DNA together with 5 μg of purified linear deletion DNA cassette. For mutant confirmation, genomic DNA was isolated from mycelia of putative deletion strains using the Wizard® Genomic DNA Purification kit (Promega). Mutant strains were confirmed by PCR through the amplification of target gene region, using primers listed in Supplementary Table 1 (IDT, Leuven, Belgium). Prior to storage, mutants were inoculated on MM plates supplemented with 1% D-glucose, 1.22 g/L uridine, and 5-fluoroorotic acid (5-FOA, Thermo Fisher Scientific) for counterselection.

2.3. Transcriptome sequencing and analysis

The transcriptomic response of *A. niger* reference strain and the Δ*galX*, Δ*xlnR*, Δ*araR*, Δ*galX*Δ*xlnR*, Δ*araR*Δ*xlnR*, Δ*araR*Δ*galX* and Δ*galX*Δ*xlnR*Δ*araR* mutants was analyzed on liquid MM supplemented with 25 mM D-glucose, 25 mM D-galactose or 5 mM L-arabinose, or a mixture of 25 mM D-galactose and 5 mM L-arabinose after 2 h induction using RNA-seq. Total RNA was extracted from grinded mycelial samples using TRIzol® reagent (Invitrogen) and purified with the NucleoSpin® RNA II Clean-up Kit (Macherey-Nagel), while contaminant genomic DNA was removed by rDNase treatment directly on the silica membrane. The RNA quality and quantity were analyzed by gel electrophoresis and NanoDrop ND-1000 (Thermo Scientific). Purification of mRNA, synthesis of cDNA library and sequencing were conducted at DOE Joint Genome Institute (JGI) as previously described (Chroumpi et al., 2020). The reads from all RNAseq samples were deposited with the Sequence Read Archive at NCBI with sample accession numbers SRP172221-172225, 172227, 172229, 172248, 172249, 172252, 172255-172264, 172266-172268, 172270, 172271, 172278, 172319-172322, 172352-172355, 172357, 172366. Statistical analysis was performed using DESeq2 (Love et al., 2014). Transcripts were considered differentially expressed if the DESeq2 fold change was > 2 or < 0.5 and Padj < 0.01 as well as the FPKM > 50 in at least one of the conditions being compared. Transcripts with FPKM ≤ 50 were considered lowly (i.e., not substantially) expressed.

2.4. Monosaccharide and polyol determination

The supernatants were heated at 95 °C for 15 min and centrifuged for 5 min at 14,000 rpm. The supernatants were 10-fold diluted with MilliQ water prior to analysis. Xylitol, L-arabitol and D-xylose were analyzed by HPLC (Dionex ICS-5000 + system; Thermo Scientific) equipped with CarboPac PA1 column (2 × 250 mm with 2 × 50 mm guard column; Thermo Scientific) as described previously (Mäkelä et al., 2016). 5–250 μM xylitol, L-arabitol and D-xylose were used as standards for identification and quantification.

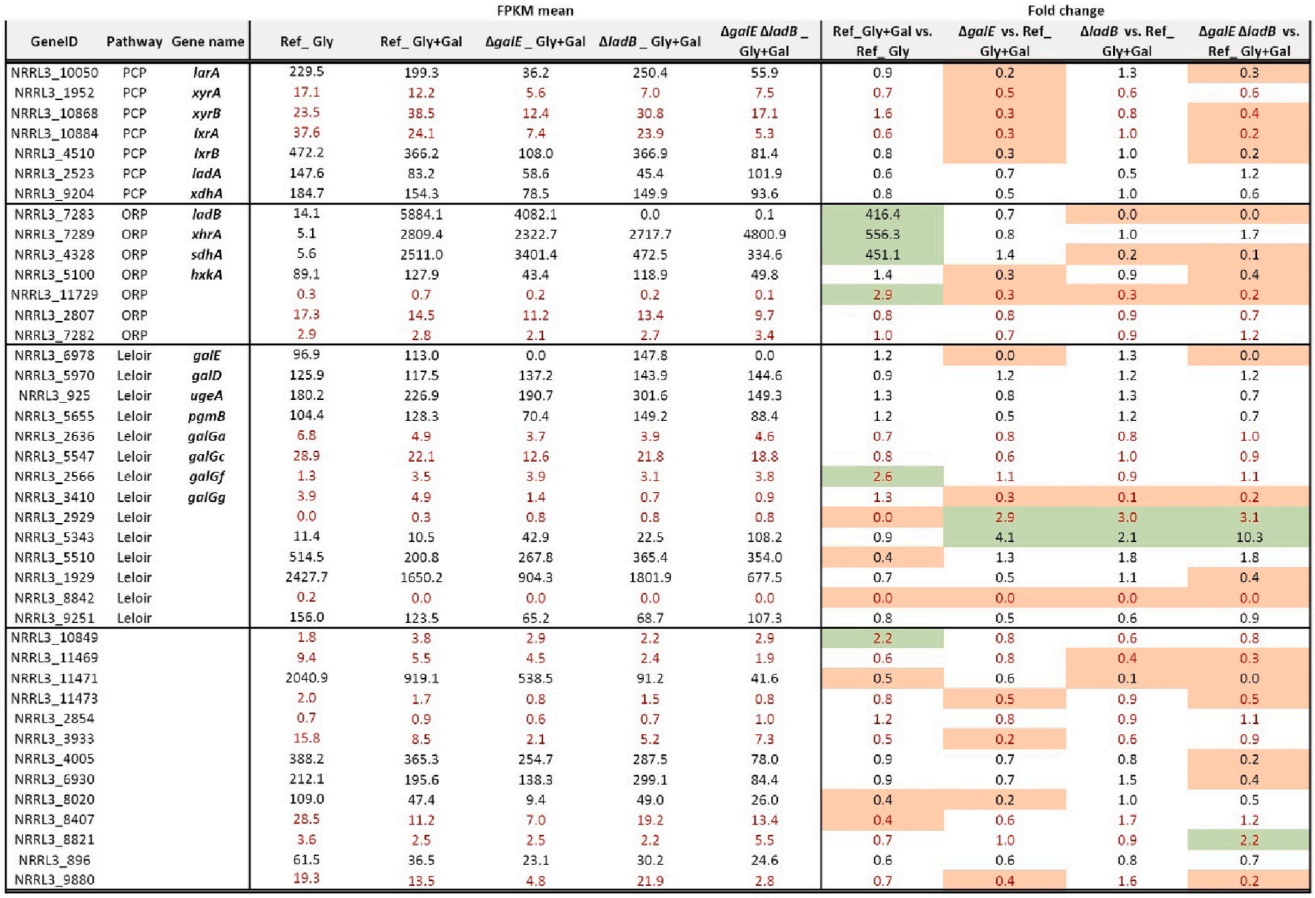
3. Results and discussion

3.1. Re-evaluation of D-galactose catabolism in A. niger reveals the involvement of XyrB and questions the contribution of SdhA in the oxido-reductive pathway

In this study, a thorough analysis of the oxido-reductive and Leloir pathways (Fig. 1) was performed in order to identify all the pathway genes, as well as to investigate the relative contribution of each pathway to D-galactose catabolism. The PCP of *A. niger* is also presented in Fig. 1, since it has been suggested that PCP genes are also involved in the D-galactose pathway. Single deletion mutants of all the already characterized and putative *A. niger* D-galactose genes were constructed to verify the phenotypes of these strains. The phenotypes were tested with both nitrate and ammonium as a nitrogen source, but this had overall no influence on the phenotype (Supplementary Fig. 1) and therefore only the nitrate medium results are discussed in detail. The transcriptome profile of the Δ*ladB*, Δ*galE* and Δ*ladB*Δ*galE* mutants, which block different parts of *A. niger* D-galactose catabolism, induced on D-galactose for 2 h was also analyzed. Since D-galactose is considered a very poor carbon source for *A. niger* (Meijer et al., 2011), glycerol was used as (additional) carbon source on both the non-induced and induced

Table 2

RNA-seq analysis of the already characterized and putative D-galactose genes in *A. niger* Δ*galE*, Δ*ladB* and Δ*ladB*Δ*galE* and the reference strains. Expression levels (FPKM) were measured after their transfer for 2 h in MM with 25 mM glycerol (Gly) or 25 mM glycerol + 25 mM D-galactose (Gly + Gal). Genes with FPKM values < 50 across all strains/conditions are considered lowly expressed and marked in red font. The values are averages of triplicates. The fold change is the difference between the deletion mutants and the reference strain. Fold changes > 2 and < 0.5 are highlighted in green and orange, respectively. PCP = Pentose catabolic pathway; ORP = oxido-reductive D-galactose catabolic pathway.



			FPKM mean					Fold change			
GeneID	Pathway	Gene name	Ref_ Gly	Ref_ Gly+Gal	Δ*galE* _ Gly+Gal	Δ*ladB* _ Gly+Gal	Δ*galE* Δ*ladB* _ Gly+Gal	Ref_Gly+Gal vs. Ref_ Gly	Δ*galE* vs. Ref_ Gly+Gal	Δ*ladB* vs. Ref_ Gly+Gal	Δ*galE* Δ*ladB* vs. Ref_ Gly+Gal
NRRL3_10050	PCP	***larA***	229.5	199.3	36.2	250.4	55.9	0.9	0.2	1.3	0.3
NRRL3_1952	PCP	***xyrA***	17.1	12.2	5.6	7.0	7.5	0.7	0.5	0.6	0.6
NRRL3_10868	PCP	***xyrB***	23.5	38.5	12.4	30.8	17.1	1.6	0.3	0.8	0.4
NRRL3_10884	PCP	***lxrA***	37.6	24.1	7.4	23.9	5.3	0.6	0.3	1.0	0.2
NRRL3_4510	PCP	***lxrB***	472.2	366.2	108.0	366.9	81.4	0.8	0.3	1.0	0.2
NRRL3_2523	PCP	***ladA***	147.6	83.2	58.6	45.4	101.9	0.6	0.7	0.5	1.2
NRRL3_9204	PCP	***xdhA***	184.7	154.3	78.5	149.9	93.6	0.8	0.5	1.0	0.6
NRRL3_7283	ORP	***ladB***	14.1	5884.1	4082.1	0.0	0.1	416.4	0.7	0.0	0.0
NRRL3_7289	ORP	***xhrA***	5.1	2809.4	2322.7	2717.7	4800.9	556.3	0.8	1.0	1.7
NRRL3_4328	ORP	***sdhA***	5.6	2511.0	3401.4	472.5	334.6	451.1	1.4	0.2	0.1
NRRL3_5100	ORP	***hxkA***	89.1	127.9	43.4	118.9	49.8	1.4	0.3	0.9	0.4
NRRL3_11729	ORP		0.3	0.7	0.2	0.2	0.1	2.9	0.3	0.3	0.2
NRRL3_2807	ORP		17.3	14.5	11.2	13.4	9.7	0.8	0.8	0.9	0.7
NRRL3_7282	ORP		2.9	2.8	2.1	2.7	3.4	1.0	0.7	0.9	1.2
NRRL3_6978	Leloir	***galE***	96.9	113.0	0.0	147.8	0.0	1.2	0.0	1.3	0.0
NRRL3_5970	Leloir	***galD***	125.9	117.5	137.2	143.9	144.6	0.9	1.2	1.2	1.2
NRRL3_925	Leloir	***ugeA***	180.2	226.9	190.7	301.6	149.3	1.3	0.8	1.3	0.7
NRRL3_5655	Leloir	***pgmB***	104.4	128.3	70.4	149.2	88.4	1.2	0.5	1.2	0.7
NRRL3_2636	Leloir	***galGa***	6.8	4.9	3.7	3.9	4.6	0.7	0.8	0.8	1.0
NRRL3_5547	Leloir	***galGc***	28.9	22.1	12.6	21.8	18.8	0.8	0.6	1.0	0.9
NRRL3_2566	Leloir	***galGf***	1.3	3.5	3.9	3.1	3.8	2.6	1.1	0.9	1.1
NRRL3_3410	Leloir	***galGg***	3.9	4.9	1.4	0.7	0.9	1.3	0.3	0.1	0.2
NRRL3_2929	Leloir		0.0	0.3	0.8	0.8	0.8	0.0	2.9	3.0	3.1
NRRL3_5343	Leloir		11.4	10.5	42.9	22.5	108.2	0.9	4.1	2.1	10.3
NRRL3_5510	Leloir		514.5	200.8	267.8	365.4	354.0	0.4	1.3	1.8	1.8
NRRL3_1929	Leloir		2427.7	1650.2	904.3	1801.9	677.5	0.7	0.5	1.1	0.4
NRRL3_8842	Leloir		0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NRRL3_9251	Leloir		156.0	123.5	65.2	68.7	107.3	0.8	0.5	0.6	0.9
NRRL3_10849			1.8	3.8	2.9	2.2	2.9	2.2	0.8	0.6	0.8
NRRL3_11469			9.4	5.5	4.5	2.4	1.9	0.6	0.8	0.4	0.3
NRRL3_11471			2040.9	919.1	538.5	91.2	41.6	0.5	0.6	0.1	0.0
NRRL3_11473			2.0	1.7	0.8	1.5	0.8	0.8	0.5	0.9	0.5
NRRL3_2854			0.7	0.9	0.6	0.7	1.0	1.2	0.8	0.9	1.1
NRRL3_3933			15.8	8.5	2.1	5.2	7.3	0.5	0.2	0.6	0.9
NRRL3_4005			388.2	365.3	254.7	287.5	78.0	0.9	0.7	0.8	0.2
NRRL3_6930			212.1	195.6	138.3	299.1	84.4	0.9	0.7	1.5	0.4
NRRL3_8020			109.0	47.4	9.4	49.0	26.0	0.4	0.2	1.0	0.5
NRRL3_8407			28.5	11.2	7.0	19.2	13.4	0.4	0.6	1.7	1.2
NRRL3_8821			3.6	2.5	2.5	2.2	5.5	0.7	1.0	0.9	2.2
NRRL3_896			61.5	36.5	23.1	30.2	24.6	0.6	0.6	0.8	0.7
NRRL3_9880			19.3	13.5	4.8	21.9	2.8	0.7	0.4	1.6	0.2

condition, in order to eliminate any stress effects due to starvation. We tested in the reference strain whether D-galactose is taken up in the presence of glycerol, and therefore could be converted intracellularly. After 2 h of incubation the initial D-galactose concentration of 25 mM decreased to 3.8 mM (data not shown), indicating that the presence of glycerol does not prevent the import of D-galactose.

3.1.1. *Phenotypic analysis of metabolic deletion mutants*

Deletion of *ladB* or *galE* did not result in significant upregulation of the Leloir and the oxido-reductive pathway genes, respectively, showing that blocking one pathway does not lead to induction of the other one (Table 2). Additionally, although previous studies indicated that D-galactose is primarily metabolized in *A. niger via* galactitol in the oxido-reductive pathway (Mojzita et al., 2012a), our data show that the dynamics between these two pathways might be different. Blocking the oxido-reductive pathway did not result in a growth arrest on D-galactose, while deletion of the Leloir pathway genes completely abolished growth on D-galactose (Fig. 2B). Any strain that contained a deletion of *galE* or *galD* was not able to grow on D-galactose. This shows that the Leloir pathway is more important than the oxido-reductive pathway in *A. niger*, suggesting that the catabolism of D-galactose is more likely facilitated through the Leloir and not the oxido-reductive pathway, at least under the tested conditions. This latter effect cannot be attributed to the toxicity of the D-galactose-1P, since deletion of *galE*, which is not expected to result in accumulation of D-galactose-1P, did also cause abolishment of growth on D-galactose (Fig. 2B). However, we should be aware that the preferential employment of either the oxido-reductive or the Leloir pathway for D-galactose catabolism could be also affected by the composition of the substrate used for the growth studies of the mutants. The Leloir pathway requires α-D-galactose, but the β-anomer is the most common form released during degradation of polysaccharides. β-D-galactose is metabolized directly by the oxido-reductive pathway. Aldose 1-epimerase, also known as mutarotase, epimerizes β-D-galactose into its α-anomer, which can further enter the Leloir pathway. The physiologically relevant mutarotase GalmB has been identified for its role in the utilization of D-galactose in *A. nidulans*, while three putative mutarotase encoding genes, NRRL3_09251, NRRL3_05510 and NRRL3_10372, have been also predicted in *A. niger* (Aguilar-Pontes et al., 2018).

Apart from its involvement in the PCP (Hasper et al., 2000) (Fig. 1), D-xylose reductase Xyl1/XyrA was previously suggested to also catalyze the conversion of D-galactose to galactitol in *Trichoderma reesei* and *A. niger* (Seiboth et al., 2007; Mojzita et al., 2012a). In *A. niger*, this conclusion was primarily based on the significantly reduced growth of the Δ*xyrA* mutant, when grown from spores on D-galactose agar plates spiked with a small amount of D-xylose (Mojzita et al., 2012a), because *A. niger* fails to germinate on D-galactose as a sole carbon source due to the absence of D-galactose transport in the conidiospores (Fekete et al., 2012). However, in our study, deletion of *xyrA* did not reduce growth on D-galactose, compared to the reference strain (Fig. 2A). In fact, both Δ*larA* and Δ*xyrA* mutants grew better on D-galactose compared to the reference strain, when mycelial plugs were used for the phenotypic

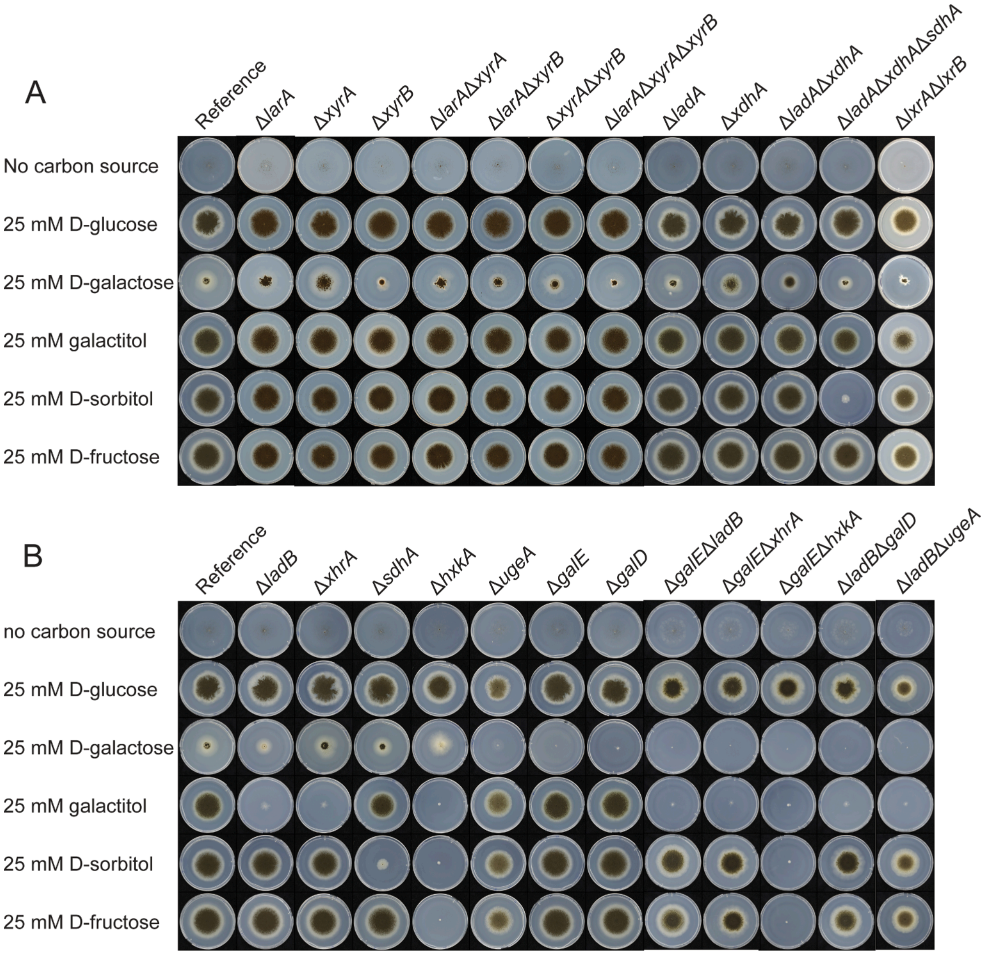


Fig. 2. (A) Growth profile of the *A. niger* pentose catabolic pathway (PCP) gene deletion mutants and (B) the oxido-reductive D-galactose pathway and Leloir pathway gene deletion mutants in comparison to the reference strain on solid MM with or without addition of carbon source. All strains were grown for 5 days at 30 °C on no carbon source, D-glucose, galactitol, D-sorbitol and D-fructose, and for 12 days at 30 °C on D-galactose. Variation in colony diameter between replicates is < 1 mm.

analysis of the mutants on agar plates. This phenotypic difference between the two studies is more likely to be related to the different growth stage of the inoculum used for the growth profiles (using spores vs. mycelia for inoculation) than to an effect of the deletions themselves.

For the single deletion mutants, slightly reduced growth was only observed in the *xyrB* deletion strain, when D-galactose was used as sole carbon source (Fig. 2A). The same pattern was also observed for the double and the triple reductase deletion mutants, when *xyrB* was absent (Fig. 2A). We recently identified *A. niger* XyrB as an additional enzyme involved in the conversion of L-arabinose and D-xylose in their polyol sugars, L-arabitol and xylitol, respectively (Chroumpi et al., 2021). However, based on the substrate specificity and catalytic efficiency of XyrB, it could also be involved in D-galactose conversion in *A. niger* (Terebieniec et al., 2021). This suggests that XyrB, and not XyrA, is involved in the *A. niger* oxido-reductive D-galactose catabolic pathway. The remaining growth of the Δ*xyrB* mutant on D-galactose could be explained by the complementary reductase activity of other enzymes catalyzing the same reaction and/or rerouting of the D-galactose catabolism towards the Leloir pathway. Deletion of the GalX-regulated gene *cipB* (NRRL3_07291), which was previously proposed as a putative D-galactose reductase that might be even more specific than XyrA (Gruben et al., 2012), did not affect growth on D-galactose or any of the oxido-reductive pathway intermediates (Supplementary Fig. 2), questioning its involvement in this pathway.

Apart from *xyrA*, several other PCP genes have been previously reported to be involved in the fungal D-galactose oxido-reductive pathway. In particular, the conversion of galactitol to L-*xylo*-3-hexulose, L-*xylo*-3-hexulose to D-sorbitol, and D-sorbitol to D-fructose were reported to be catalyzed by the L-arabitol dehydrogenase Lad1 (Pail et al., 2004), the L-*xylo*-3-hexulose reductase Lxr4 and the xylitol dehydrogenase Xdh1, respectively, in *T. reesei* (Seiboth et al., 2007; Seiboth and Metz, 2011). The last conversion was also suggested for *A. nidulans* (Flipphi et al., 2009), but in this species, it is still unclear if L-*xylo*-3-hexulose needs to be converted to L-sorbose before it can be converted to D-sorbitol. The conversion of L-sorbose to D-sorbitol was suggested to be catalyzed by the L-xylulose reductase LxrA (Seiboth and Metz, 2011). In our study, deletion of both *A. niger* L-xylulose reductase encoding genes, *lxrA* and *lxrB*, did not result in reduced growth on D-galactose or any of the other tested D-galactose pathway intermediates (Fig. 2). The same was observed for deletion of L-arabitol and xylitol dehydrogenases, *ladA* and *xdhA*. This is consistent with the fact that one of these PCP genes was induced in the presence of D-galactose (Table 2).

Deletion of the *A. niger* galactitol dehydrogenase encoding gene *ladB* resulted in growth arrest on galactitol and significantly reduced growth on D-galactose (Fig. 2B), as previously reported by Mojzita et al. (2012a). Deletion of *xhrA*, which has been shown to be involved in the conversion of L-*xylo*-3-hexulose to D-sorbitol in *A. niger* (Mojzita et al., 2012b), caused abolishment of growth on galactitol, while growth on D-galactose was similar to the reference strain (Fig. 2B). Similarly, deletion of *hxkA*, which catalyzes the last step of the oxido-reductive D-galactose catabolic pathway, resulted in abolishment of growth on the pathway intermediates galactitol, D-sorbitol and D-fructose (Fig. 2B). However, this gene was not shown to be upregulated on D-galactose, compared to glycerol (Table 2). This is likely due to the involvement of HxkA in other catabolic pathways, such as glycolysis and D-mannose catabolic pathways (Panneman et al., 1998). Deletion of the more downstream pathway genes, *xhrA* and *hxkA*, did not result in similar reduction of growth on D-galactose (Fig. 2B), which implies that this either redirects D-galactose to the Leloir pathway or that other enzymes contribute to these steps.

3.1.2. Transcriptome comparison of the metabolic mutants and the reference strain

Based on our transcriptome data (Table 2), both oxido-reductive and Leloir pathway genes seem to be induced by D-galactose and not another pathway intermediate, as deletion of *ladB* and *galE* did not result in reduced expression levels of these genes in the presence of D-galactose (Table 2). In these strains, the formation of pathway intermediates is blocked and therefore these cannot be the inducers of the pathway genes. Interestingly, there is also no upregulation of the Leloir pathway genes in the *ladB* mutant or of the oxido-reductive pathway genes in the *galE* mutant, indicating that *A. niger* does not try to compensate for the block of one pathway by upregulation of the genes of the other pathway.

However, it should be noted that the expression levels of the Leloir genes were significantly lower compared to the oxido-reductive pathway genes on D-galactose (Table 2), which is surprising, considering the stronger effect of blocking the Leloir pathway on growth on D-galactose. Interestingly, *sdhA* does not appear to be co-regulated with the other pathway genes, since it seems to be induced by D-sorbitol and not D-galactose. The *sdhA* gene seems to be downregulated in the absence of D-sorbitol, since deletion of *ladB* resulted in its reduced expression on D-galactose (Table 2; FPKM mutants vs Ref on D-galactose). Deletion of *sdhA* resulted in growth arrest on D-sorbitol, but did not significantly affect growth on galactitol (Fig. 2B), as also previously reported (Koivistoinen et al., 2012). Our hypothesis is that another D-sorbitol dehydrogenase is involved in the oxido-reductive D-galactose pathway, which has higher affinity for D-sorbitol. Biochemical analysis of SdhA indicates that it is a relatively low affinity D-sorbitol dehydrogenase ($K_m = 50$ mM) (Koivistoinen et al., 2012), which makes it doubtful that it could efficiently convert the low levels of D-sorbitol that would be present during growth on D-galactose. During growth on higher concentrations of D-sorbitol, SdhA would be required. This could explain the phenotype of the Δ*sdhA* mutant on galactitol as well as the abolishment of growth on D-sorbitol. Apart from *sdhA*, *deh1* (NRRL3_7284), *gutB* (NRRL3_01929) and NRRL3_08842 have also been previously predicted as putative *A. niger* D-sorbitol dehydrogenase encoding genes (Gruben et al., 2012; Aguilar-Pontes, 2018). However, deletion of the GalX regulated gene *deh1* did not affect growth on D-galactose or any of the oxido-reductive pathway intermediates, including D-sorbitol (Supplementary Fig. 2). Additionally, NRRL3_08842 was not significantly expressed under the tested conditions, while *gutB* was highly expressed on all conditions tested, but not specifically induced on D-galactose (data not shown). Nevertheless, their involvement in the pathway still requires further physiological investigation.

3.2. GalX, AraR and XlnR contribute to the regulation of D-galactose and L-arabinose catabolism in A. niger

To investigate the involvement and contribution of AraR, XlnR and GalX in D-galactose and pentose catabolism, as well as to identify any cross-regulation or co-regulation relationships between the different TFs, we constructed single, double and triple gene deletion combinations of these regulators. The transcriptomic response of these regulatory mutants induced after 2 h on D-glucose, L-arabinose and D-galactose, and a mixture of 25 mM D-galactose and 5 mM L-arabinose was also analyzed to support the phenotypic data. We tested whether the concentrations of both sugars were reduced but still present after 2 h incubation in the reference strain, as this would ensure that both D-galactose and L-arabinose responsive induction would then be possible. The initial concentration of 25 mM D-galactose and 2 mM L-arabinose reduced to 3.9 mM and 1.0 mM (data not shown), respectively, demonstrating that both sugars are taken up, but D-galactose to a higher extent. This confirms a previous study in which the consumption of a mixture of nine sugars was analyzed in *A. niger* (Mäkelä et al., 2018), and in which D-galactose uptake largely preceded L-arabinose uptake.

The growth profiles of the deletion strains were analyzed on all the sugars and intermediates of the PCP and oxido-reductive D-galactose pathway (Fig. 3). A mixture of 25 mM D-galactose and 2 mM L-arabinose (Gal + Ara) was also used to evaluate the role of L-arabinose activation on D-galactose uptake in *A. niger*. Although 2 mM of L-arabinose, when

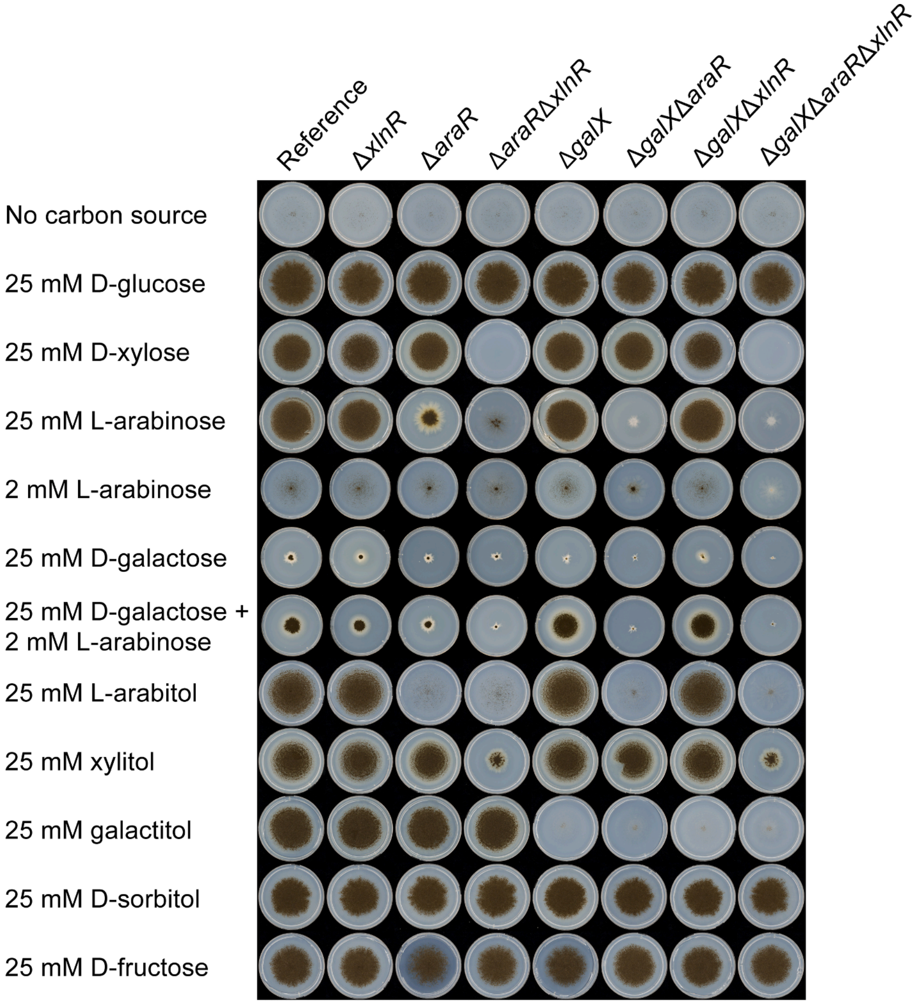


Fig. 3. Growth profile of the *A. niger* reference strain and araR, xlnR and galX single and combinatorial gene deletion mutants on solid MM with or without addition of carbon source. All TF mutants were grown for 10 days at 30 °C on no carbon source, D-glucose, D-xylose, L-arabinose, L-arabitol, xylitol, galactitol, D-sorbitol and D-fructose, and for 12 days at 30 °C on D-galactose, and the mixture of D-galactose and L-arabinose. Variation in colony diameter between replicates is $<$ 1 mm.

used as a sole carbon source, was not sufficient to promote significant growth of the *A. niger* strains (Fig. 3), it was shown to be sufficient to induce AraR (J. Meng and R.P. de Vries, unpublished data).

Single deletion of the genes coding for the arabinanolytic (AraR) and (hemi-)cellulolytic (XlnR) regulators did not significantly affect growth on D-xylose (Fig. 3), as previously reported (Battaglia et al., 2011a). However, growth was abolished when both *araR* and *xlnR* were deleted, showing that they can compensate for each other's absence during growth on D-xylose. Although, we cannot exclude that the expression of the D-xylose catabolic genes is also affected by other regulators, these putative regulators cannot compensate for the loss of AraR and XlnR.

While the growth of the single deletion mutants Δ*araR* and Δ*xlnR* was not affected on xylitol, the growth of the double Δ*araR*Δ*xlnR* deletion mutant was significantly reduced but not abolished. This shows that probably other TFs also activate expression of the genes involved in xylitol conversion, or that xylitol catabolism can be also facilitated through an alternative route. However, at the moment, there is no evidence for the presence of an alternative pathway for xylitol catabolism in *A. niger*. Three enzymes are involved in xylitol conversion in *A. niger*: the L-arabitol dehydrogenase LadA, the xylitol dehydrogenase XdhA and the D-sorbitol dehydrogenase SdhA (Chroumpi et al., 2021). In *A. niger*, *ladA* is regulated by AraR, while *xdhA* is regulated by both AraR and XlnR (Battaglia et al., 2011a), which was confirmed by our transcriptome data. Based on our expression data, *sdhA* was significantly upregulated on D-galactose, while deletion of *galX* and of *xlnR* resulted in significant downregulation of *sdhA*, but only a small effect was observed in Δ*araR* (Table 3). This indicates that both GalX and XlnR, and to a lesser extent AraR, are involved in the regulation of *sdhA*. However, deletion of *galX* in the Δ*araR*Δ*xlnR* mutant did not further reduce growth on xylitol (Fig. 3), which demonstrates that the conversion of this compound is not affected by GalX. Finally, the unaffected growth of all tested regulatory mutants on D-sorbitol, supports our hypothesis about the involvement of an alternative D-sorbitol dehydrogenase in D-galactose catabolism in *A. niger*.

Deletion of *araR* reduced growth on L-arabinose, while combined deletion of *araR* and *xlnR* caused an even stronger growth impairment compared to the Δ*araR* mutant (Fig. 3), as previously reported (Battaglia et al., 2011b). This more severe effect observed in Δ*araR*Δ*xlnR* can be attributed to the co-regulation of the L-arabinose catabolic pathway by AraR and XlnR. Interestingly, deletion of *galX* in the Δ*araR* background resulted in further reduced growth compared to the single Δ*araR* and the double Δ*araR*Δ*xlnR* mutants on L-arabinose (Fig. 3), showing that GalX also affects L-arabinose catabolism in *A. niger*. However, this effect was only present when both *araR* and *galX* genes were deleted. In the single Δ*galX* mutant, no growth reduction was observed on L-arabinose (Fig. 3). Since growth of Δ*galX* and Δ*araR*Δ*galX* mutants was not affected on any other L-arabinose pathway intermediates than L-arabinose, GalX is likely involved in the initial step of the pathway in which L-arabinose is converted to L-arabitol (Fig. 1). However, *galX* deletion does not seem to affect the expression of *larA*, *xyrA* and *xyrB* on L-arabinose

Table 3

RNA-seq analysis of the genes involved in the PCP and D-galactose pathways in *A. niger* Δ*araR*, Δ*xlnR*, Δ*galX*, Δ*araR*Δ*xlnR*, Δ*galX*Δ*xlnR*, Δ*galX*Δ*araR*, Δ*galX*Δ*araR*Δ*xlnR* and the reference strains. For all strains, expression levels (FPKM) were measured after their transfer for 2 h in MM with 25 mM D-glucose, 5 mM L-arabinose or 25 mM D-galactose, or 25 mM D-galactose + 5 mM L-arabinose. Genes with FPKM values < 50 are considered lowly expressed and marked in red font. The values are averages of triplicates. The fold change is the difference between the deletion mutants and the reference strain. Fold changes > 2 and < 0.5 are highlighted in green and orange, respectively.

				FPKM mean								Fold Change						
	GeneID	Pathway	Gene name	Ref	Δ*galX*	Δ*araR*	Δ*galX*Δ*araR*	Δ*xlnR*	Δ*galX*Δ*xlnR*	Δ*araR*Δ*xlnR*	Δ*galX*Δ*araR*Δ*xlnR*	Δ*galX* vs. Ref	Δ*araR* vs. Ref	Δ*galX*Δ*araR* vs. Ref	Δ*xlnR* vs. Ref	Δ*galX*Δ*xlnR* vs. Ref	Δ*araR*Δ*xlnR* vs. Ref	Δ*galX*Δ*araR*Δ*xlnR* vs. Ref
25 mM D-glucose	NRRL3_5970	Leloir	*galD*	208.1	189.7	303.2	216.5	185.9	83.6	29.0	60.6	0.9	1.5	1.0	0.9	0.4	0.1	0.3
	NRRL3_6978	Leloir	*galE*	181.9	179.4	152.3	177.5	130.0	89.1	52.1	82.3	1.0	0.8	1.0	0.7	0.5	0.3	0.5
	NRRL3_925	Leloir	*ugeA*	214.6	301.2	290.0	252.6	222.5	188.0	143.6	196.5	1.4	1.4	1.2	1.0	0.9	0.7	0.9
	NRRL3_5100	ORP	*hxkA*	207.5	217.5	104.7	141.4	80.7	183.1	145.2	139.5	1.0	0.5	0.7	0.4	0.9	0.7	0.7
	NRRL3_4328	ORP	*sdhA*	4.8	11.6	741.9	14.1	7.9	3.3	2.0	3.5	2.4	153.4	2.9	1.6	0.7	0.4	0.7
	NRRL3_7283	ORP	*ladB*	0.9	1.9	1.6	1.1	0.5	0.9	0.2	0.6	2.2	1.8	1.2	0.6	1.0	0.2	0.7
	NRRL3_7289	ORP	*xhrA*	0.6	30.4	16.5	20.2	0.3	26.3	0.1	11.7	52.5	28.4	34.8	0.5	45.3	0.2	20.2
	NRRL3_10868	PCP	*xyrB*	36.5	59.2	50.2	47.2	92.5	56.2	29.4	31.9	1.6	1.4	1.3	2.5	1.5	0.8	0.9
	NRRL3_10884	PCP	*lxrA*	47.7	69.0	430.5	11.8	21.2	72.9	16.3	19.5	1.4	9.0	0.2	0.4	1.5	0.3	0.4
	NRRL3_1952	PCP	*xyrA*	6.1	8.0	326.4	4.3	2.3	4.2	3.9	4.2	1.3	53.8	0.7	0.4	0.7	0.6	0.7
	NRRL3_2523	PCP	*ladA*	55.9	96.4	734.5	17.3	33.3	62.9	23.6	24.9	1.7	13.1	0.3	0.6	1.1	0.4	0.4
	NRRL3_4471	PCP	*xkiA*	25.7	27.3	302.4	33.1	46.0	40.0	19.9	26.6	1.1	11.8	1.3	1.8	1.6	0.8	1.0
	NRRL3_4510	PCP	*lxrB*	1412.9	1474.5	982.4	1463.2	924.8	786.3	705.1	674.0	1.0	0.7	1.0	0.7	0.6	0.5	0.5
	NRRL3_9204	PCP	*xdhA*	106.6	133.5	587.3	47.1	83.0	102.0	62.3	72.7	1.3	5.5	0.4	0.8	1.0	0.6	0.7
	NRRL3_10050	PCP	*larA*	246.9	387.6	1138.9	71.1	81.3	100.0	37.3	23.0	1.6	4.6	0.3	0.3	0.4	0.2	0.1
5 mM L-arabinose	NRRL3_5970	Leloir	*galD*	500.9	396.7	121.9	252.6	675.2	710.9	87.6	143.5	0.8	0.2	0.5	1.3	1.4	0.2	0.3
	NRRL3_6978	Leloir	*galE*	203.3	189.0	67.7	89.7	217.0	247.8	62.4	47.5	0.9	0.3	0.4	1.1	1.2	0.3	0.2
	NRRL3_925	Leloir	*ugeA*	571.6	682.2	86.0	82.1	476.8	362.0	125.0	107.3	1.2	0.2	0.1	0.8	0.6	0.2	0.2
	NRRL3_5100	ORP	*hxkA*	42.9	51.8	17.0	24.9	37.6	42.0	33.9	27.0	1.2	0.4	0.6	0.9	1.0	0.8	0.6
	NRRL3_4328	ORP	*sdhA*	14.1	6.6	2.9	1.8	2.9	2.8	2.0	2.6	0.5	0.2	0.1	0.2	0.2	0.1	0.2
	NRRL3_7283	ORP	*ladB*	1.5	2.4	5.7	7.3	6.4	5.1	4.1	6.6	1.6	3.7	4.9	4.2	3.4	2.7	4.4
	NRRL3_7289	ORP	*xhrA*	0.4	28.1	0.8	38.2	1.2	41.7	0.4	26.8	66.4	1.8	90.2	2.9	98.5	0.9	63.2
	NRRL3_10868	PCP	*xyrB*	531.1	463.2	189.1	208.4	81.4	94.3	96.6	78.0	0.9	0.4	0.4	0.2	0.2	0.2	0.1
	NRRL3_10884	PCP	*lxrA*	2777.1	2711.4	11.7	16.2	2358.5	2239.2	18.7	16.0	1.0	0.0	0.0	0.8	0.8	0.0	0.0
	NRRL3_1952	PCP	*xyrA*	3325.5	3237.4	2290.9	2236.5	1668.5	1277.1	5.4	4.9	1.0	0.7	0.7	0.5	0.4	0.0	0.0
	NRRL3_2523	PCP	*ladA*	3610.6	4837.6	263.9	214.4	3445.7	2635.7	122.1	118.4	1.3	0.1	0.1	1.0	0.7	0.0	0.0
	NRRL3_4471	PCP	*xkiA*	1653.4	1411.5	74.9	111.5	1045.8	856.3	24.4	23.4	0.9	0.0	0.1	0.6	0.5	0.0	0.0
	NRRL3_4510	PCP	*lxrB*	128.7	184.2	328.4	322.4	319.7	314.6	482.3	365.0	1.4	2.6	2.5	2.5	2.4	3.7	2.8
	NRRL3_9204	PCP	*xdhA*	5176.4	5277.7	1241.1	1082.8	7604.9	5769.5	259.5	251.7	1.0	0.2	0.2	1.5	1.1	0.1	0.0
	NRRL3_10050	PCP	*larA*	5492.7	5956.0	61.0	112.4	4999.7	3986.2	89.2	77.7	1.1	0.0	0.0	0.9	0.7	0.0	0.0
25 mM D-galactose	NRRL3_5970	Leloir	*galD*	75.1	63.3	57.9	128.6	145.9	110.3	59.9	89.0	0.8	0.8	1.7	1.9	1.5	0.8	1.2
	NRRL3_6978	Leloir	*galE*	70.1	77.4	50.0	65.3	79.5	89.1	53.6	44.0	1.1	0.7	0.9	1.1	1.3	0.8	0.6
	NRRL3_925	Leloir	*ugeA*	119.7	105.6	111.3	151.5	143.9	127.2	126.8	154.4	0.9	0.9	1.3	1.2	1.1	1.1	1.3
	NRRL3_5100	ORP	*hxkA*	43.6	49.3	43.5	66.9	59.1	85.2	69.2	45.2	1.1	1.0	1.5	1.4	2.0	1.6	1.0
	NRRL3_4328	ORP	*sdhA*	3923.7	430.3	2687.3	38.8	517.2	15.8	854.1	55.1	0.1	0.7	0.0	0.1	0.0	0.2	0.0
	NRRL3_7283	ORP	*ladB*	6802.1	7.3	6915.6	5.8	4785.2	8.6	6066.5	4.0	0.0	1.0	0.0	0.7	0.0	0.9	0.0
	NRRL3_7289	ORP	*xhrA*	3373.8	47.3	3232.2	66.8	2169.0	52.6	3035.1	44.3	0.0	1.0	0.0	0.6	0.0	0.9	0.0
	NRRL3_10868	PCP	*xyrB*	9.4	7.6	14.2	10.8	43.4	55.1	30.6	13.4	0.8	1.5	1.2	4.6	5.9	3.3	1.4
	NRRL3_10884	PCP	*lxrA*	5.6	9.0	6.8	11.2	62.8	203.4	33.8	8.6	1.6	1.2	2.0	11.2	36.2	6.0	1.5
	NRRL3_1952	PCP	*xyrA*	11.5	13.7	12.3	7.8	3.9	3.5	5.1	3.5	1.2	1.1	0.7	0.3	0.3	0.4	0.3
	NRRL3_2523	PCP	*ladA*	66.3	89.0	79.9	57.1	141.2	176.5	108.5	57.5	1.3	1.2	0.9	2.1	2.7	1.6	0.9
	NRRL3_4471	PCP	*xkiA*	15.1	15.8	16.1	42.3	74.9	77.1	72.5	91.2	1.0	1.1	2.8	5.0	5.1	4.8	6.1
	NRRL3_4510	PCP	*lxrB*	90.5	117.9	169.8	294.3	397.0	378.9	184.5	105.4	1.3	1.9	3.3	4.4	4.2	2.0	1.2
	NRRL3_9204	PCP	*xdhA*	117.9	157.0	130.5	113.5	181.4	172.4	144.1	120.4	1.3	1.1	1.0	1.5	1.5	1.2	1.0
	NRRL3_10050	PCP	*larA*	25.8	41.9	37.0	54.2	117.6	138.3	66.7	19.0	1.6	1.4	2.1	4.6	5.4	2.6	0.7
25 mM D-galactose + 5 mM L-arabinose	NRRL3_5970	Leloir	*galD*	501.3	507.4	149.5	265.7	417.6	393.6	65.1	114.4	1.0	0.3	0.5	0.8	0.8	0.1	0.2
	NRRL3_6978	Leloir	*galE*	183.2	189.3	65.9	85.9	119.2	143.6	54.2	53.5	1.0	0.4	0.5	0.7	0.8	0.3	0.3
	NRRL3_925	Leloir	*ugeA*	342.2	328.2	129.7	141.9	188.9	148.9	118.9	173.9	1.0	0.4	0.4	0.6	0.4	0.3	0.5
	NRRL3_5100	ORP	*hxkA*	49.1	51.5	42.2	63.7	44.7	77.1	61.4	51.5	1.0	0.9	1.3	0.9	1.6	1.3	1.0
	NRRL3_4328	ORP	*sdhA*	4053.0	2150.6	2242.0	44.8	782.4	56.6	1038.9	55.2	0.5	0.6	0.0	0.2	0.0	0.3	0.0
	NRRL3_7283	ORP	*ladB*	5541.7	4.2	5735.6	5.8	3836.8	6.8	6065.8	4.1	0.0	1.0	0.0	0.7	0.0	1.1	0.0
	NRRL3_7289	ORP	*xhrA*	2935.7	46.8	2790.5	55.5	1634.3	51.5	3104.8	47.4	0.0	1.0	0.0	0.6	0.0	1.1	0.0
	NRRL3_10868	PCP	*xyrB*	120.4	65.3	33.6	33.2	38.1	52.9	24.8	16.3	0.5	0.3	0.3	0.3	0.4	0.2	0.1
	NRRL3_10884	PCP	*lxrA*	1203.4	1256.9	5.7	16.2	338.1	892.4	30.3	12.0	1.0	0.0	0.0	0.3	0.7	0.0	0.0
	NRRL3_1952	PCP	*xyrA*	955.4	969.5	542.8	361.0	33.3	37.7	3.4	2.7	1.0	0.6	0.4	0.0	0.0	0.0	0.0
	NRRL3_2523	PCP	*ladA*	2124.9	2137.4	96.1	87.6	516.3	680.2	89.0	64.6	1.0	0.0	0.0	0.2	0.3	0.0	0.0
	NRRL3_4471	PCP	*xkiA*	897.9	852.7	67.4	110.5	298.4	311.6	75.4	108.8	0.9	0.1	0.1	0.3	0.3	0.1	0.1
	NRRL3_4510	PCP	*lxrB*	75.5	101.8	136.4	267.7	274.7	296.1	141.6	117.9	1.3	1.8	3.5	3.6	3.9	1.9	1.6
	NRRL3_9204	PCP	*xdhA*	1533.7	1660.8	297.5	250.9	522.3	597.4	141.8	111.7	1.1	0.2	0.2	0.3	0.4	0.1	0.1
	NRRL3_10050	PCP	*larA*	3050.3	3133.9	31.5	73.1	518.9	622.1	59.6	33.5	1.0	0.0	0.0	0.2	0.2	0.0	0.0

(Table 3), suggesting that its influence may be through the activation of other reductases that can also convert L-arabinose or possibly through D-galactose transport.

Phenotypic and gene expression analysis of a *galX* disruptant mutant (Δ*galX*) has previously revealed that GalX regulates the D-galactose oxido-reductive pathway, but not the Leloir pathway in *A. niger* (Gruben et al., 2012). As expected, deletion of *galX* did not affect the expression of the Leloir pathway genes (Table 3). Our data revealed that AraR, XlnR and GalX are all involved in the catabolism of D-galactose in *A. niger*, but with a different relative contribution and role during growth on D-galactose. The first three steps of the oxido-reductive D-galactose catabolic pathway, namely the conversions of D-galactose to galactitol, galactitol to L-*xylo*-3-hexulose and L-*xylo*-3-hexulose to D-sorbitol (Fig. 1), seem to be indeed regulated by GalX. Deletion of *galX* reduced growth on D-galactose, while the growth on galactitol was completely abolished in the Δ*galX* mutant (Fig. 3). As discussed earlier, XyrB is also involved in the first step of the *A. niger* oxido-reductive D-galactose catabolic pathway (Fig. 1), which could explain the residual growth of the Δ*galX* mutant on D-galactose (Fig. 3). The *A. niger* D-xylose reductase encoding gene *xyrB* has been previously shown to be upregulated on L-arabinose and D-xylose (Chroumpi et al., 2021), while *araR* and *xlnR* deletions negatively affected *xyrB* expression (Table 3), indicating its regulation by both AraR and XlnR. Supporting that, deletion of all three TFs resulted in abolished growth of the Δ*galX*Δ*araR*Δ*xlnR* mutant on both pentose sugars as well as on D-galactose (Fig. 3). However, the conversion of D-sorbitol to D-fructose and D-fructose to D-fructose-1P do not seem to be under the regulation of any of the three tested TFs, as also shown in a previous study (Gruben et al., 2012).

In contrast to the oxido-reductive pathway genes, the Leloir pathway genes *galE*, *galD* and *ugeA* were significantly upregulated on L-arabinose compared to D-galactose and D-glucose (Table 3). Furthermore, deletion of *araR* resulted in their downregulation, indicating that the Leloir pathway is controlled by AraR, and as a result, L-arabinose induced. D-galactose and L-arabinose are commonly present together in plant biomass and share many biochemical structural features, which could explain this organization of the regulatory network. Since 2 mM of L-arabinose was not sufficient to promote significant growth of the *A. niger* strains (Fig. 3), but only induce AraR, the reduced growth of Δ*araR* on the Gal + Ara mixture (Fig. 3) can be explained by the downregulation of Leloir pathway. Unexpectedly, the growth of the Δ*galX* mutant on the Gal + Ara mixture was significantly improved compared to the reference strain (Fig. 3). This phenotype was not due to carbon repression, because deletion of the CreA regulator did not affect the phenotype (Supplementary Fig. 3), but could probably be explained by (indirect) repression of the Leloir pathway genes by GalX. In this case, the reduced repression effect in the absence of GalX could justify the better growth of the Δ*galX* mutant under these conditions. However, this result is opposite to a previous study, where the deletion of the *galX* gene in *A. niger* has led to growth reduction on a mixture of 25 mM D-galactose and 3 mM L-arabinose compared to the reference strain (Gruben et al., 2012).

Finally, no significant changes could be observed in the expression levels of genes involved in D-galactose release in the Δ*galX* strain compared to the reference strain (Supplementary Table 2A). This shows that GalX does not control D-galactose release from oligo- and polysaccharides, as also previously shown by Gruben et al. (2012). Here as well, most genes encoding α- and β-galactosidases, and endo- and exo-galactanases (den Herder et al., 1992; Kumar et al., 1992; de Vries et al., 1999; Ademark et al., 2001; de Vries et al., 2002; Coutinho et al., 2009) were neither specifically induced on D-galactose nor significantly downregulated in the Δ*galX* mutant compared to the reference strain (Supplementary Table 2B). However, an *exo*-1,6-galactanase (NRRL3_08701), two β-galactosidases (NRRL3_02630 and NRRL3_02479) and an α-galactosidase (NRRL3_00743) were specifically induced on L-arabinose, and their expression was significantly reduced in the Δ*araR* mutant.

4. Conclusions

In *A. niger*, pentose and D-galactose catabolism were revealed to be more interconnected than previously shown, at both the metabolic and regulatory level. Enzymes that have been previously shown to be involved in the PCP also participate in D-galactose catabolism, and vice versa, while three TFs, GalX, AraR and XlnR, contribute to the regulation of D-galactose and L-arabinose catabolism in *A. niger*. Since L-arabinose, D-xylose and D-galactose are often simultaneously present in the native environment of *A. niger* this has probably stimulated the evolution of this interconnected regulatory system. Finally, this study clearly emphasizes the necessity for better characterization of the sugar catabolic genes, as well as their regulatory mechanisms, in order to allow for more efficient design of fungal cell factories.

CRediT authorship contribution statement

Tania Chroumpi: Methodology, Formal analysis, Investigation, Writing – original draft. **Natalia Martínez-Reyes:** Formal analysis, Investigation. **Roland S. Kun:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Mao Peng:** Formal analysis, Writing – review & editing. **Anna Lipzen:** Data curation, Formal analysis. **Vivian Ng:** Project administration. **Sravanthi Tejomurthula:** Investigation, Formal analysis. **Yu Zhang:** Investigation, Formal analysis. **Igor V. Grigoriev:** Supervision. **Miia R. Mäkelä:** Supervision, Writing – review & editing. **Ronald P. de Vries:** Formal analysis, Resources, Funding acquisition, Supervision, Writing – review & editing. **Sandra Garrigues:** Investigation, Formal analysis, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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References

Ademark, P., de Vries, R.P., Hägglund, P., Stålbrand, H., Visser, J., 2001. Cloning and characterization of *Aspergillus niger* genes encoding an α-galactosidase and a β-mannosidase involved in galactomannan degradation. Eur. J. Biochem. 268, 2982–2990. https://doi.org/10.1046/j.1432-1327.2001.02188.x.

Aguilar-Pontes, M.V., Brandl, J., McDonnell, E., Strasser, K., Nguyen, T.T.M., Riley, R., et al., 2018. The gold-standard genome of *Aspergillus niger* NRRL 3 enables a detailed view of the diversity of sugar catabolism in fungi. Stud. Mycol. 91, 61–78. https://doi.org/10.1016/j.simyco.2018.10.001.

Aguilar-Pontes, M.V., 2018. Tracing the fungal carbon metabolic roadmap. In: *Utrecht University Repository (Dissertation)*.

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

Battaglia, E., Hansen, S.F., Leendertse, A., Madrid, S., Mulder, H., Nikolaev, I., et al., 2011b. Regulation of pentose utilisation by AraR, but not XlnR, differs in *Aspergillus nidulans* and *Aspergillus niger*. Appl. Microbiol. Biotechnol. 91, 387–397. https://doi.org/10.1007/s00253-011-3242-2.

Battaglia, E., Zhou, M., de Vries, R.P., 2014. The transcriptional activators AraR and XlnR from *Aspergillus niger* regulate expression of pentose catabolic and pentose phosphate pathway genes. Res. J. Microbiol. 165, 531–540. https://doi.org/10.1016/j.resmic.2014.07.013.

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

Brown, N.A., Ries, L.N.A., Goldman, G.H., 2014. How nutritional status signalling coordinates metabolism and lignocellulolytic enzyme secretion. Fungal Genet. Biol. 72, 48–63. https://doi.org/10.1016/j.fgb.2014.06.012.

Chroumpi, T., Aguilar-Pontes, M.V., Peng, M., Wang, M., Lipzen, A., Ng, V., et al., 2020. Identification of a gene encoding the last step of the L-rhamnose catabolic pathway in *Aspergillus niger* revealed the inducer of the pathway regulator. Microbiol. Res. 234, 126426 https://doi.org/10.1016/j.micres.2020.126426.

Chroumpi, T., Peng, M., Aguilar-Pontes, M.V., Müller, A., Wang, M., Yan, J., et al., 2021. Revisiting a ’simple’ fungal metabolic pathway reveals redundancy, complexity and diversity. Microb. Biotechnol. https://doi.org/10.1111/1751-7915.13790.

Coutinho, P.M., Andersen, M.R., Kolenova, K., vanKuyk, P.A., Benoit, I., Gruben, B.S., et al., 2009. Post-genomic insights into the plant polysaccharide degradation potential of *Aspergillus nidulans* and comparison to *Aspergillus niger* and *Aspergillus oryzae*. Fungal Genet. Biol. 46, S161–S169. https://doi.org/10.1016/j.fgb.2008.07.020.

de Groot, M.J.L., van den Dool, C., Wosten, H.A.B., Levisson, M., vanKuyk, P.A., Ruijter, G.J.G., et al., 2007. Regulation of pentose catabolic pathway genes of *Aspergillus niger*. Food Technol. Biotechnol. 45, 134–138.

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

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

de Vries, R.P., Parenicová, L., Hinz, S.W., Kester, H.C., Beldman, G., Benen, J.A., et al., 2002. The β-1,4-endogalactanase A gene from *Aspergillus niger* is specifically induced on arabinose and galacturonic acid and plays an important role in the degradation of pectic hairy regions. Eur. J. Biochem. 269, 4985–4993. https://doi.org/10.1046/j.1432-1033.2002.03199.x.

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

de Vries, R.P., Patyshakuliyeva, A., Garrigues, S., Agarwal-Jans, S., 2020. The current biotechnological status and potential of plant and algal biomass degrading/modifying enzymes from ascomycete fungi. In: Nevalainen, H. (Ed.), Grand Challenges in Fungal Biotechnology. Springer International Publishing, Cham, pp. 81–120.

den Herder, I.F., Mateo Rosell, A.M., van Zuilen, C.M., Punt, P.J., van den Hondel, C.A.M. J.J., 1992. Cloning and expression of a member of the *Aspergillus niger* gene family encoding α-galactosidase. Mol. Gen. Genet. 233, 404–410. https://doi.org/10.1007/BF00265437.

Doench, J.G., Hartenian, E., Graham, D.B., Tothova, Z., Hegde, M., Smith, I., et al., 2014. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nat. Biotechnol. 32, 1262–1267. https://doi.org/10.1038/nbt.3026.

Fekete, E., Karaffa, L., Sándor, E., Bányai, I., Seiboth, B., Gyémánt, G., et al., 2004. The alternative D-galactose degrading pathway of *Aspergillus nidulans* proceeds via L-sorbose. Arch. Microbiol. 181, 35–44. https://doi.org/10.1007/s00203-003-0622-8.

Fekete, E., de Vries, R.P., Seiboth, B., vanKuyk, P.A., Sándor, E., Fekete, É., et al., 2012. D-Galactose uptake is nonfunctional in the conidiospores of *Aspergillus niger*. FEMS Microbiol. Lett. 329, 198–203. https://doi.org/10.1111/j.1574-6968.2012.02524.x.

Flipphi, M., Sun, J., Robellet, X., Karaffa, L., Fekete, E., Zeng, A.-P., et al., 2009. Biodiversity and evolution of primary carbon metabolism in *Aspergillus nidulans* and other *Aspergillus* spp. Fungal Genet. Biol. 46, S19–S44. https://doi.org/10.1016/j.fgb.2008.07.018.

Frey, P.A., 1996. The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. FASEB J. 10, 461–470. https://doi.org/10.1096/fasebj.10.4.8647345.

Gruben, B.S., Zhou, M., de Vries, R.P., 2012. GalX regulates the D-galactose oxido-reductive pathway in *Aspergillus niger*. FEBS Lett. 586, 3980–3985. https://doi.org/10.1016/j.febslet.2012.09.029.

Hasper, A.A., Visser, J., de Graaff, L.H., 2000. The *Aspergillus niger* transcriptional activator XlnR, which is involved in the degradation of the polysaccharides xylan and cellulose, also regulates D-xylose reductase gene expression. Mol. Microbiol. 36, 193–200. https://doi.org/10.1046/j.1365-2958.2000.01843.x.

Hasper, A.A., Trindade, L.M., van der Veen, D., van Ooyen, A.J.J., de Graaff, L.H., 2004. Functional analysis of the transcriptional activator XlnR from *Aspergillus niger*. Microbiology 150, 1367–1375. https://doi.org/10.1099/mic.0.26557-0.

Khosravi, C., Benocci, T., Battaglia, E., Benoit, I., de Vries, R.P., 2015. Sugar catabolism in *Aspergillus* and other fungi related to the utilization of plant biomass. Adv. Appl. Microbiol. 90, 1–28. https://doi.org/10.1016/bs.aambs.2014.09.005.

Koivistoinen, O.M., Richard, P., Penttilä, M., Ruohonen, L., Mojzita, D., 2012. Sorbitol dehydrogenase of *Aspergillus niger*, SdhA, is part of the oxido-reductive D-galactose pathway and essential for D-sorbitol catabolism. FEBS Lett. 586, 378–383. https://doi.org/10.1016/j.febslet.2012.01.004.

Kowalczyk, J.E., Benoit, I., de Vries, R.P., 2014. Regulation of plant biomass utilization in *Aspergillus*. Adv. Appl. Microbiol. 88, 31–56. https://doi.org/10.1016/B978-0-12-800260-5.00002-4.

Kowalczyk, J.E., Gruben, B.S., Battaglia, E., Wiebenga, A., Majoor, E., de Vries, R.P., 2015. Genetic interaction of *Aspergillus nidulans* GalR, XlnR and AraR in regulating D-galactose and L-arabinose release and catabolism gene expression. PLoS ONE 10. https://doi.org/10.1371/journal.pone.0143200.

Kumar, V., Ramakrishnan, S., Teeri, T.T., Knowles, J.K.C., Hartley, B.S., 1992. *Saccharomyces cerevisiae* cells secreting an *Aspergillus niger* β-galactosidase grow on whey permeate. Nat. Biotechnol. 10, 82–85. https://doi.org/10.1038/nbt0192-82.

Kun, R.S., Meng, J., Salazar-Cerezo, S., Mäkelä, M.R., de Vries, R.P., Garrigues, S., 2020. CRISPR/Cas9 facilitates rapid generation of constitutive forms of transcription factors in *Aspergillus niger* through specific on-site genomic mutations resulting in increased saccharification of plant biomass. Enzyme Microb. Tech. 136, 109508 https://doi.org/10.1016/j.enzmictec.2020.109508.

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

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8.

Mäkelä, M.R., Mansouri, S., Wiebenga, A., Rytioja, J., de Vries, R.P., Hildén, K.S., 2016. *Penicillium subrubescens* is a promising alternative for *Aspergillus niger* in enzymatic plant biomass saccharification. N. Biotechnol. 33, 834–841. https://doi.org/10.1016/j.nbt.2016.07.014.

Mäkelä, M.R., Aguilar-Pontes, M.V., van Rossen-Uffink, D., Peng, M., de Vries, R.P., 2018. The fungus *Aspergillus niger* consumes sugars in a sequential manner that is not mediated by the carbon catabolite repressor CreA. Sci. Rep. 8, 6655. https://doi.org/10.1038/s41598-018-25152-x.

Meijer, M., Houbraken, J.A.M.P., Dalhuijsen, S., Samson, R.A., de Vries, R.P., 2011. Growth and hydrolase profiles can be used as characteristics to distinguish *Aspergillus niger* and other black aspergilli. Stud. Mycol. 69, 19–30. https://doi.org/10.3114/sim.2011.69.02.

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

Mojzita, D., Koivistoinen, O.M., Maaheimo, H., Penttilä, M., Ruohonen, L., Richard, P., 2012a. Identification of the galactitol dehydrogenase, LadB, that is part of the oxido-reductive D-galactose catabolic pathway in *Aspergillus niger*. Fungal Genet. Biol. 49, 152–159. https://doi.org/10.1016/j.fgb.2011.11.005.

Mojzita, D., Herold, S., Metz, B., Seiboth, B., Richard, P., 2012b. L-xylo-3-hexulose reductase is the missing link in the oxidoreductive pathway for D-galactose catabolism in filamentous fungi. J. Biol. Chem. 287, 26010–26018. https://doi.org/10.1074/jbc.M112.372755.

Németh, Z., Kulcsár, L., Flipphi, M., Orosz, A., Aguilar-Pontes, M.V., de Vries, R.P., et al., 2019. L-Arabinose induces D-galactose catabolism *via* the Leloir pathway in *Aspergillus nidulans*. Fungal Genet. Biol. 123, 53–59. https://doi.org/10.1016/j.fgb.2018.11.004.

Pail, M., Peterbauer, T., Seiboth, B., Hametner, C., Druzhinina, I., Kubicek, C.P., 2004. The metabolic role and evolution of L-arabinitol 4-dehydrogenase of *Hypocrea jecorina*. Eur. J. Biochem. 271, 1864–1872. https://doi.org/10.1111/j.1432-1033.2004.04088.x.

Panneman, H., Ruijter, G.J.G., van den Broeck, H.C., Visser, J., 1998. Cloning and biochemical characterisation of *Aspergillus niger* hexokinase. FEBS J. 258, 223–232. https://doi.org/10.1046/j.1432-1327.1998.2580223.x.

Peng, M., Khosravi, C., Lubbers, R.J.M., Kun, R.S., Aguilar Pontes, M.V., Battaglia, E., et al., 2021. CreA-mediated repression of gene expression occurs at low monosaccharide levels during fungal plant biomass conversion in a time and substrate dependent manner. Cell Surf. 7, 100050 https://doi.org/10.1016/j.tcsw.2021.100050.

Ronne, H., 1995. Glucose repression in fungi. Trends Genet. 11, 12–17. https://doi.org/10.1016/s0168-9525(00)88980-5.

Ruijter, G.J., Vanhanen, S.A., Gielkens, M.M., van de Vondervoort, P.J., Visser, J., 1997. Isolation of *Aspergillus niger creA* mutants and effects of the mutations on expression of arabinases and L-arabinose catabolic enzymes. Microbiology 143, 2991–2998. https://doi.org/10.1099/00221287-143-9-2991.

Seiboth, B., Hartl, L., Pail, M., Fekete, E., Karaffa, L., Kubicek, C.P., 2004. The galactokinase of *Hypocrea jecorina* is essential for cellulase induction by lactose but dispensable for growth on D-galactose. Mol. Microbiol. 51, 1015–1025. https://doi.org/10.1046/j.1365-2958.2003.03901.x.

Seiboth, B., Gamauf, C., Pail, M., Hartl, L., Kubicek, C.P., 2007. The D-xylose reductase of *Hypocrea jecorina* is the major aldose reductase in pentose and D-galactose catabolism and necessary for β-galactosidase and cellulase induction by lactose. Mol. Microbiol. 66, 890–900. https://doi.org/10.1111/j.1365-2958.2007.05953.x.

Seiboth, B., Metz, B., 2011. Fungal arabinan and L-arabinose metabolism. Appl. Microbiol. Biotechnol. 89 (6), 1665–1673. https://doi.org/10.1007/s00253-010-3071-8.

Song, L., Ouedraogo, J.P., Kolbusz, M., Nguyen, T.T.M., Tsang, A., 2018. Efficient genome editing using tRNA promoter-driven CRISPR/Cas9 gRNA in *Aspergillus niger*. PLoS ONE 13, e0202868. https://doi.org/10.1371/journal.pone.0202868.

Strauss, J., Horvath, H.K., Abdallah, B.M., Kindermann, J., Mach, R.L., Kubicek, C.P., 1999. The function of CreA, the carbon catabolite repressor of *Aspergillus nidulans*, is regulated at the transcriptional and post-transcriptional level. Mol. Microbiol. 32, 169–178. https://doi.org/10.1046/j.1365-2958.1999.01341.x.

Terebieniec, A., Chroumpi, T., Dilokpimol, A., Aguilar-Pontes, M.V., Mäkelä, M.R., de Vries, R.P., 2021. Characterization of D-xylose reductase, XyrB, from *Aspergillus niger*. Biotechnol. Rep. 30, e00610 https://doi.org/10.1016/j.btre.2021.e00610.

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

Vesth, T.C., Nybo, J.L., Theobald, S., Frisvad, J.C., Larsen, T.O., Nielsen, K.F., et al., 2018. Investigation of inter- and intraspecies variation through genome sequencing of *Aspergillus* section *Nigri*. Nat. Genet. 50, 1688–1695. https://doi.org/10.1038/s41588-018-0246-1.