RESEARCH ARTICLE

Genetic Interaction of Aspergillus nidulans galR, xlnR and araR in Regulating D-Galactose and L-Arabinose Release and Catabolism Gene Expression

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Abstract

In Aspergillus nidulans, the xylanolytic regulator XlnR and the arabinanolytic regulator AraR co-regulate pentose catabolism. In nature, the pentose sugars D-xylose and L-arabinose are both main building blocks of the polysaccharide arabinoxylan. In pectin and arabinogalactan, these two monosaccharides are found in combination with D-galactose. GalR, the regulator that responds to the presence of D-galactose, regulates the D-galactose catabolic pathway. In this study we investigated the possible interaction between XlnR, AraR and GalR in pentose and/or D-galactose catabolism in A. nidulans. Growth phenotypes and metabolic gene expression profiles were studied in single, double and triple disruptant A. nidulans strains of the genes encoding these paralogous transcription factors. Our results demonstrate that AraR and XlnR not only control pentose catabolic pathway genes, but also genes of the oxido-reductive D-galactose catabolic pathway. Conversely, GalR is not involved in regulation of pentose catabolism, but controls only genes of the oxido-reductive D-galactose catabolic pathway.

Introduction

Filamentous fungi are able to degrade structural plant cell wall polysaccharides (e.g. xylan and pectin) and catabolize the released monosaccharides [1]. The production of the enzymes needed for polysaccharide degradation is controlled by transcriptional regulators. Some of these regulators have been identified and characterized in Aspergillus, such as the transcriptional activators AraR, GalR and XlnR [2–4]. These regulators are most likely induced by L-arabinose/L-arabitol, D-galactose and D-xylose, respectively, and also control the metabolic conversion of these monosaccharides. This provides the fungus with a quick and controllable system that responds to the polymers present in the environment. Notably, these
monosaccharides often co-occur in nature, in particular in plant biomass. Xylan, the most abundant hemicellulose in hardwoods and cereals, has a backbone of $\beta-1,4$-linked D-xylose units that can be branched with L-arabinose and D-galactose, but also acetyl, feruloyl, p-coumaric and D-gluconic acid [1]. Arabinogalactan, commonly found in softwoods like larch, is made of branched D-galactose units with L-arabinose side chains. Xyloglucan is a polysaccharide present in all terrestrial plants [5]. It consists of $\beta-1,4$-linked D-glucose chains that can be, depending on the type, substituted with D-xylose, L-arabinose and D-galactose. The primary cell wall of terrestrial plants is also rich in pectin [6]. Pectin is a complex polymer build from four structural elements: homogalacturonan, xyloglacturonan, and rhamnogalacturonan (I and II). Rhamnogalacturonan-I part has a backbone of alternating D-galacturonic acid and L-rhamnose residues, with side chains that contains significant amounts of L-arabinose and/or D-galactose. The xylogalacturonan part of pectin consists of a D-galacturonic acid backbone with $\beta$-linked residues of D-xylose. These common plant polysaccharides provide a simultaneous source of D-xylose, L-arabinose and D-galactose for the fungus. Therefore, the intracellular pathways that convert them could also work simultaneously.

The pentose catabolic pathway (PCP) converts both L-arabinose and D-xylose to D-xylulose-5-phosphate, which then enters the pentose phosphate pathway (PPP) [7]. In L-arabinose catabolism, L-arabinose is converted to L-arabitol, L-xylulose and xylitol by L-arabinose reductase (LarA), L-arabitol-4-dehydrogenase (LadA) and L-xylulose reductase (LxrA), respectively (Fig 1a) [8–10]. D-xylose is converted to xylitol by L-xylulose reductase (XyrA) [11]. Xylitol is then converted to D-xylulose and D-xylulose-5-phosphate by xylitol dehydrogenase (XdhA) and D-xylulose kinase (XkiA), respectively [9,12]. AraR is the main regulator of the PCP genes, with the exception of XyrA which is under control of XlnR [3]. AraR is present only in Aspergilli and other species from the order Eurotiiales while XlnR is present in most Ascomycetes [3].

Filamentous fungi use several pathways for D-galactose catabolism. The best studied pathway is the Leloir pathway, which is present in prokaryotic and eukaryotic organisms [14]. In A. nidulans the first step of the Leloir pathway is a conversion of D-galactose to D-galactose-1-phosphate catalyzed by galactokinase (GalE) (Fig 1b). The second step is catalyzed by D-galactose-1-phosphate-uridylotransferase (GalD) and generates two products, UDP-galactose and D-glucose-1-phosphate. UDP-galactose can then be re-cycled to UDP-glucose by UDP-galactose-4-epimerase (GalG) and D-glucose-1-phosphate to D-glucose-6-phosphate by phosphoglucomutase (PgmB) [14–16].

A. nidulans also possesses an oxido-reductive D-galactose catabolic pathway that converts D-galactose to galactitol, which is then converted to L-sorbose [17]. L-sorbose is reduced to D-sorbitol that in turn is converted to D-fructose and D-fructose-1-phosphate (Fig 1c). Not all enzymes that catalyze particular steps in A. nidulans have been identified but similarity to T. reesei was suggested [4,17]. In T. reesei the oxido-reductive D-galactose conversion involves three enzymes of the PCP: Xyl1, Lad1 and Xdh1 (Fig 1c). Aldose reductase (Xyl1) is the major enzyme that catalyzes the reduction of D-galactose as well as reduction of L-arabinose and D-xylose [18]. L-arabitol dehydrogenase (Lad1) catalyzes conversion of both L-arabitol to L-xylulose [19] and galactitol to L-xylo-3-hexulose [20] and the corresponding gene is upregulated in the presence of L-arabinose and D-galactose [20]. Xylitol dehydrogenase (Xdh1) was proposed to catalyze oxidation of both xylitol and sorbitol [13].

Distinct enzymes are involved in some enzymatic steps of the oxido-reductive D-galactose catabolic pathway in A. niger. The ladA gene of A. niger, homologous to T. reesei lad1, was induced on L-arabinose but not on D-galactose or galactitol [21]. A. niger has a separate galactitol dehydrogenase gene, ladB, catalyzing the conversion of galactitol to L-xylo-3-hexulose [21]. This gene is also present in A. nidulans. The next step, reduction of L-xylo-3-hexulose to
D-sorbitol is catalyzed by L-xylo-3-hexulose reductase, XhrA, not by LxrA in *A. niger* [13].

Conversion of D-sorbitol to D-fructose is catalyzed by a sorbitol dehydrogenase (SdhA) [22] (Fig 1c). The first step of this conversion pathway, D-galactose reduction, also involves D-xylose reductase (XyrA) from PCP, as was shown in *T. reesei* [21].

The regulation of D-galactose catabolic pathways in *A. nidulans* remains unclear, although some interesting indications for interactions between the D-galactose-responsive regulator, GalR, and arabinanolytic regulator, AraR, have been suggested. In the previous study [4], a model of D-galactose catabolism in *A. nidulans* and *T. reesei* was presented in which LadA (in principal involved in PCP) was predicted to catalyze also D-galactitol conversion in D-galactitol oxido-reductive pathway. Moreover, the expression of ladA was reported to be controlled by GalR on D-galactose [4] while expression of this gene on L-arabinose is known to be under control of AraR [3]. In *A. nidulans* two transcriptional factors (TFs), GalR and GalX, which respond to the presence of D-galactose were identified [4]. GalX is present in most *Aspergillus* species, while GalR was described as specific for *A. nidulans* [4]. The deletion of galR in *A. nidulans or galX in A. nidulans and A. niger causes reduced growth on D-galactose and galactitol and several genes of oxido-reductive and Leloir pathways seem to be affected by this mutation [4,23].

In this study, we investigated the genetic interaction between XlnR, AraR and GalR in detail by studying the phenotype of single, double and triple disruptants of genes encoding these regulators in *A. nidulans*. Moreover, we present the influence of those mutations on the expression of genes from pentose and D-galactose catabolism.
Material and Methods
Strains, culture conditions and media

*A. nidulans* strains (Table 1) were grown at 37°C using minimal medium (MM) or complete medium (CM) [24]. Plates of these media contained 1.5% agar. Spore plates contained CM + 2% glucose, while plates used in growth experiments contained MM + 25 mM monosaccharide or 1% polysaccharide. The pH of the medium containing apple pectin was adjusted to 6. The supplements arginine or uridine were added (0.2 g L⁻¹ and 1.2 g L⁻¹, respectively) when required. Liquid cultures were grown on a rotary shaker at 250 rpm. Pre-cultures for RNA isolation were grown in 1 L Erlenmeyer flasks containing 250 ml CM with 2% D-fructose. After 16h of incubation, the mycelium was harvested, washed with MM and transferred to 250 ml Erlenmeyer flasks containing 50 ml MM + 25 mM carbon source (D-fructose, D-xylose, L-arabinose or D-galactose). After 2 h of incubation, the mycelium was harvested by vacuum filtration, dried between tissue paper and frozen in liquid nitrogen. New mutant strains were deposited at CBS Fungal Biodiversity Centre with strain numbers indicated in Table 1.

Sexual crosses

Sexual crosses between ΔxlnR, ΔaraR and ΔgalR strains were performed as described previously [25]. Strains were screened by selecting for poor growth on L-arabinose, D-xylose and/or D-galactose. Absence of the regulatory genes was verified by Southern blot analysis.

Molecular biology methods

Molecular biology methods were performed according to standard procedures [26], unless stated otherwise. For gDNA and RNA isolation frozen mycelium was ground using a TissueLyser II (QIAGen). RNA was extracted using TRIzol reagent (Invitrogen) [27] and purified with NucleoSpin RNA II Clean-up kit (Macherey-Nagel) with DNase treatment. The RNA quantity of the samples was checked with a NanoDrop-1000 spectrophotometer and the quality by RNA gel electrophoresis. Total RNA in amount of 2.5 μg was reverse transcribed using the ThermoScript™ RT-PCR System (Invitrogen, Carlsbad, USA) and obtained cDNA was diluted 100x and used in the qRT-PCR reaction.

Gene expression assays

Gene expression was assayed by quantitative real-time PCR (qRT-PCR; Applied Biosystems 7500 Real-time PCR system) using ABI Fast SYBR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers were designed using PrimerExpress™ 3.0 software (Applied Biosystems, Foster City, CA, USA) according to the supplier’s instructions (Table 2) and validated experimentally. Optimal primer concentrations were determined by analysis of dissociation curves for amplicons generated by combinations of 50nM, 300nM and 900nM (final concentrations) primer per pair. The efficiency of optimal primer pairs was assessed using the correlation between the cycle threshold value (Ct) and the logarithm of *A. niger* gDNA dilutions (10ng to 1pg). The amplification efficiency for all the primer couples was estimated at 91–101%. The qRT-PCR reaction contained 10μl ABI Fast SYBR Master Mix, 2μl forward and reverse primer (at optimal concentration) and 2μl of cDNA in the final volume of 20μl. The cycling conditions were 95°C for 20sec, followed by 40 cycles of 95°C for 3sec and 60°C for 30sec. A dissociation curve was generated to confirm that single product was amplified. Amplification quality was verified by using No Template Control (NTC), which contained 2μl of water instead of cDNA. Expression levels were normalized against β-tubulin (tubC) as a physiological reference and calculated according to the relative quantification 2⁻ΔCt method [28].
Two biological and three technical replicates were analyzed. GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) was used to calculate the mean, standard deviation and significance of the samples. Statistical significance was determined using multiple unpaired two-tailed $t$-tests and corrected with Holm-Sidak method, with $P$-value < 0.05.

Genomic cluster comparison

Synteny of gal$X$ and gal$R$ genes in the genome of $A. nidulans$, $A. versicolor$, $A. sydowii$, $A. fumigatus$ A1163, $A. fumigatus$ A293, $A. flavus$, $A. oryzae$, $A. niger$ CBS 513.88, $A. niger$ ATCC 1015, $A. acidus$, $A. kawachii$, $A. brasiliensis$, $A. carbonarius$, $A. wentii$ and $A. glaucus$ was analyzed using the Sybil algorithm [29] at the Aspergillus Genome Database (AspGD; http://www.aspergillusgenome.org).

### Table 1. $A. nidulans$ strains used in this study.

| Strain | Code | Strain number | Genotype | Reference |
|--------|------|---------------|----------|-----------|
| AN031  | Ref  | CBS 129193    | pyrG8, argB2 | Battaglia, 2011a |
| ΔaraR  | ΔA   | CBS 129196    | pyrG89, argB2, ΔaraR::pyrG+ | Battaglia, 2011a |
| ΔxlnR  | ΔX   | CBS 129195    | pyrG89, argB2, ΔxlnR::pyrB+ | Battaglia, 2011a |
| ΔgalR  | ΔG   | CBS 138911    | pyrG89, argB2, ΔgalR::pyrG+ | Battaglia, 2011a |
| ΔxlnR/ΔaraR | ΔXΔA | CBS 129197    | pyrG89, argB2, ΔaraR::pyrG+, ΔxlnR::pyrB+ | This study |
| ΔxlnR/ΔgalR | ΔXΔG | CBS 138912    | pyrG89, argB2, ΔxlnR::argB+, ΔgalR::pyrG+ | This study |
| ΔaraR/ΔgalR | ΔAΔG | CBS 138910    | pyrG89, argB2, ΔaraR::pyrG+, ΔgalR::pyrG+ | This study |
| ΔaraR/ΔgalR | ΔAΔΔ | CBS 138909    | pyrG89, argB2, ΔaraR::pyrG+, ΔgalR::pyrG+ | This study |

### Table 2. Primers used in this study to generate the gene fragments for qRT-PCR analysis (a) and Southern Blot (b).

#### a)

| Gene | Gene number | Forward | Reverse |
|------|-------------|---------|---------|
| tubC | AN6838      | CGGAAACCTGGCGCTCAATAT | CCACCACCGATCCGACACT |
| xlnR | AN7610      | CGCCGCTTCAATCACACT | CGGCTTGGATGTTGAGAATCTAGA |
| araR | AN0388      | GTCCGGCACCTTCCGAGC | GCAACGAAAGGAGCCAT |
| galR | AN10550     | TCGCTTACACTGACTAATGAGA | GCGCTGGCGCATTTATCA |
| larA | AN7193      | GAGGCCAATCCGGTACT | TGGAAATTTCGCCGAGATG |
| ladA | AN0942      | ATCCGAGCTTGAACATTTTG | TCCCGCGTCAGCTAG |
| lxR | AN10169     | TGCCCGGATAGTGAATCTA | GCGCTTGGCCGATAG |
| xdR | AN9064      | GTGCTGATGTCGGGATGAT | CATGGAATCCGGTGAGAATCA |
| xkiA | AN8790      | AACAGACCCGCGCATGAG | GAGACGGAAGAGGCAT |
| xyrA | AN0423      | ACTCGTGTGTTGGCGCC | GTCCTCCGCCGCTTTCAG |
| galD | AN6182      | CCTGGCCAAACCAAATGAGA | TGGCGGCGCCCTTGT |
| galE | AN4957      | CAGGCTGGGAGAATAC | CAGGCTGGTGGTGAAGCAGG |
| galF | AN9148      | CCTGGGCGCCGTTGTG | CAGGGTGGTGGTGAAGCAGG |
| ladB | AN4336      | CGGGTCAGACAGTCATTCTC | CCCCGTGCAAGACATAG |
| sdhA | AN2666      | ACCCCGCAATTCTTTCTC | CGACCTGGCGACATAG |
| hxA | AN7459      | TGCCGACAGGTTTCGACAT | TCAAGAGGGCGGACTAG |

#### b)

| Gene | Gene number | Forward | Reverse |
|------|-------------|---------|---------|
| xlnR | AN7610      | GCAATCGTGCGAGATG | TGGATTTCGCCGAGAC |
| araR | AN0388      | ACGATGGGGAATCCCATCACCC | GTGGAACAGATCCGTCG |
| galR | AN10550     | TGGACACACAGGCTGGCAG | TCGAAATCCAGGAAGAT |

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Results and Discussion

In *A. nidulans*, two transcription factors (TFs) co-regulate genes of the pentose catabolic pathway (XlnR and AraR) and two TFs co-regulate genes of the galactose conversion pathways (GalR and GalX) [3,4]. In this study we investigated possible genetic interaction between three paralogous sugar-specific TFs (XlnR, AraR and GalR) in *A. nidulans*. The full length protein sequences of XlnR, AraR and GalR share more than 30% amino acid sequence identity (Fig 2) and are relatively conserved in both the DNA binding and activation domains [3,4]. Within the Zn$_2$Cys$_6$ binuclear DNA binding domain, AraR shares 51% and 63% sequence identity with XlnR and GalR, respectively (Fig 3, Table 3). Detailed protein alignments of TF pairs can be found in the supplementary data (S1 Fig). Co-involvement of AraR and XlnR in regulation of the PCP and PPP has been described [3,30], while the genetic interactions between AraR and XlnR was never addressed before. The higher level of sequence conservation in the DNA binding domains of AraR and GalR suggest they may also co-regulate the same target genes. Less conservation was observed within the DNA binding domain of GalR and XlnR (Table 3; 44% identity), which is similar to the conservation degree between the non-paralogous TFs XlnR and InuR (43% identity). Despite the lower degree of conservation between these two paralogs, we cannot exclude possible genetic interaction between galR and xlnR. In this study we will elucidate co-regulation between all three TFs in the PCP and galactose conversion pathways. We constructed double and triple regulator *A. nidulans* mutants to study their growth phenotype on different carbon sources and expression levels of metabolic genes.

Growth of the *A. nidulans* reference strain, and single (ΔX, ΔA and ΔG), double (ΔXΔA, ΔXΔG, ΔAΔG), and triple (ΔΔΔ) disruptants (see Table 1 for strain designations) was compared on different sugars (mono- and polysaccharides) with fructose as a reference carbon source (Fig 4). The influence of the nitrogen source was tested by growing the strains in the presence of two different nitrogen sources, ammonium and nitrate respectively, but no differences in growth on the carbon sources were observed (data not shown). Expression of orthologous genes involved in PCP and oxido-reductive D-galactose catabolic pathway in *T. reesei* (larA, ladA, lxrA, xyrA, xdhA, xkiA), and from the oxido-reductive D-galactose pathway (ladB, sdhA, hxaA) and Leloir pathway (galE, galD, galP) in *A. niger* was analyzed by qRT-PCR after growth in media containing D-fructose, D-xylose, L-arabinose or D-galactose as a carbon source. Expression of araR, xlnR and galR was measured to confirm the deletion of the regulator in the respective strains. In addition, this was used to determine how the regulator encoding genes respond to the disruption of each other (Fig 5).

Growth of *A. nidulans* on L-arabinose and L-arabitol was reduced in all strains in which araR is absent (Fig 4). This correlates with the expression results which showed that the first three genes involved in PCP (larA, ladA, lxrA) are under control of AraR as they are significantly down regulated in all ΔaraR strains on L-arabinose (Fig 6a). The expression of those genes on D-fructose is absent and it is induced on L-arabinose (Fig 6a). This confirms the previously described role of AraR as the major regulator of the PCP genes, and in particular its influence on enzymatic steps that are required for L-arabinose conversion [3]. The growth on arabinan was decreased for the strains in which araR was absent (Fig 4). This confirms that the role of AraR extends to regulation of expression of genes encoding extracellular enzymes. This phenomenon was already described in *A. niger*, in which expression of the α-arabinofuranosidase encoding genes abfA and abfB are under regulatory control of AraR [3].

Growth of *A. nidulans* on D-xylose and xylitol was only affected when both xlnR and araR are deleted (Fig 4). The last two enzymes of the PCP (xdhA, xkiA) and D-xylose reductase (xyrA) are co-regulated by XlnR and AraR and only the ΔxlnR ΔaraR double mutant showed significantly reduced expression on L-arabinose (Fig 6b). Reduced expression of xyrA gene in
ΔAAΔX interrupted conversion of D-xylose via the pentose catabolic pathway and caused the observed growth reduction. Similarly, reduced expression of xdhA gene in ΔAAΔX caused reduced growth on xylitol (Fig 4). Interestingly expression patterns of those XlnR and AraR co-regulated genes were observed in single mutants. The xdhA and xkiA genes were highly expressed in the absence of XlnR and not reduced in the absence of AraR. The xyrA gene

Fig 2. Amino acid sequence alignment of A. nidulans GalR, AraR and XlnR. The alignment was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) and visualized using Easy Sequencing in PostScript (http://espript.ibcp.fr/ESPript/ESPript/index.php). Conserved regions are marked by shaded boxes and similar regions by unshaded boxes. The Zn_{2}Cys_{6} binuclear DNA binding domain is underlined and the six conserved cysteine residues are indicated by stars.
shows the opposite pattern, it is not reduced in the absence of XlnR and highly expressed in the absence of AraR. These observations indicate which TF is the predominate activator of the pentose catabolic pathway gene (Table 4). All the genes involved in the PCP in A. nidulans showed low expression in triple and some double knockout strains on L-arabinose (Fig 6a and 6b). This observed low level of expression may be a basal constitutive expression. A similar low expression level was seen in the reference strain on D-fructose, which is not an inducer of the pentose catabolic pathway (Fig 6a and 6b). On beechwood xylan, reduction in colony density but not in the diameter was observed in all strains in which xlnR was absent (Fig 4). The growth of single, double and triple mutants of xlnR, araR and galR was not affected on galactan (galactose), apple pectin (D-galacturonic acid, rhamnose, arabinose, galactose, xylose), arabinogalactan (arabinose, galactose) and guar gum (mannose, galactose) (Fig 4). This demonstrated that the influence of those mutations is mainly on intracellular level.

It was previously suggested that the conversion of galactitol to L-sorbose could be catalyzed by L-arabitol dehydrogenase, LadA, which is normally involved in arabitol oxidation in A. nidulans [17]. The expression of ladA was reported to be controlled by GalR [4]. To test the hypothesis that ladA and the other enzymes of the PCP could be under regulation of GalR in A. nidulans, we analyzed the expression of larA, ladA, lxrA, xdh, xkiA and xyrA on both L-arabinose and D-galactose. The expression of larA, ladA and lxrA was significantly lower on D-galactose compared to L-arabinose in the reference strain (Fig 6a). Similar to previous

Table 3. Amino acid sequence identity (%) within the Zn2Cys6 binuclear DNA binding domain of GalR, AraR and XlnR in A. nidulans. InuR is an example of non-paralogous TF.

|            | GalR (AN10550) | AraR (AN0388) | XlnR (AN7610) | InuR (AN3835) |
|------------|----------------|---------------|---------------|---------------|
| GalR (AN10550) | -              |               |               |               |
| AraR (AN0388)  | 63% (27/43)   | -             |               |               |
| XlnR (AN7610)  | 44% (17/39)   | 51% (20/39)   | -             |               |
| InuR (AN3835)  | 29% (11/38)   | 39% (15/38)   | 43% (17/40)   | -             |

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results, the PCP genes are induced on L-arabinose compared to D-fructose. However, most of the PCP genes seem not to be induced on D-galactose and show only a basal level of expression. Moreover, deletion of galR did not reduce the expression of larA, ladA, lxrA, xdhA, xkiA and xyrA on D-galactose and L-arabinose (Fig 6a and 6b). Therefore, we conclude that GarR is not involved in regulation of expression of PCP genes on D-galactose and L-arabinose. In contrast,
our data suggest that both AraR and XlnR are involved in regulation of D-galactose oxido-reductive catabolic pathway (see below).

Growth on D-galactose was reduced when GalR is absent, suggesting that GalR regulates the genes involved in D-galactose conversion (Fig 4) [4]. A previous study suggested that GalR is regulating the expression of galactokinase gene galE which converts D-galactose to D-galactose-1-phosphate in the Leloir pathway [4]. We compared expression levels of genes involved...
in the Leloir and the oxido-reductive D-galactose catabolic pathways in galR mutant and wild type grown of D-galactose as a carbon source. Expression of the Leloir pathway genes galD, galE and galF was not affected by the absence of GalR (Fig 7a) and therefore it cannot explain the observed growth reduction. In the alternative oxido-reductive D-galactose catabolic pathway D-galactose is converted to D-galactitol by a yet unknown aldose reductase. To explain the observed growth reduction in ΔG, we hypothesize that this unknown enzyme is regulated by
GalR. It is worth to notice that the growth was highly reduced but not abolished in ΔG on D-galactose (Fig 4). The residual growth of ΔG could be caused by D-galactose catabolized through the unaffected Leloir pathway, but it also suggests that the oxido-reductive pathway is the preferred D-galactose conversion road in \textit{A. nidulans}.

Growth on D-galactitol was slightly reduced when \textit{araR} and/or \textit{galR} were absent (Fig 4). Galactitol is an intermediate product in the oxido-reductive D-galactose pathway. Our findings negate the involvement of GalR-regulated \textit{ladA} in conversion of galactitol. We deduced that \textit{A. nidulans} similarly to \textit{A. niger} has evolved distinct enzyme sets that catalyze the PCP and the D-galactose oxido-reductive pathway. The \textit{ladB} gene has recently been characterized as a galactitol dehydrogenase encoding gene in \textit{A. niger} \cite{21}. In this study we tested the expression of the \textit{ladB} ortholog in \textit{A. nidulans}. Expression of \textit{ladB} on D-galactose was high in the reference strain, significantly down regulated in ΔXΔG, and almost absent in ΔXΔA and ΔXΔΔ (Fig 7b). This suggests that both XlnR and AraR (and to some point XlnR and GalR) are controlling \textit{ladB}, but it does not explain what causes the growth reduction observed in single \textit{araR} and \textit{galR} mutants on galactitol. In the ΔXΔG double mutant, AraR is still present and probably compensates for the loss of the other two, which makes the change in expression less extreme (Fig 7b). Although there is no difference in \textit{ladB} expression between the reference strain and the ΔA on D-galactose, a strong reduction was observed when comparing \textit{ladB} expression in the double mutant to the ΔA and ΔG single mutants. The fold change difference between ΔA and ΔAΔG is significant which means that the absence of GalR was (partially) responsible for this reduction (Fig 7b). A similar effect was observed when comparing ΔG and ΔAΔG. Altogether, the expression of \textit{ladB} in the regulatory mutants illustrates high complexity of gene co-regulation. A previous study \cite{4} suggested that \textit{galR} and \textit{ladB} are under control of GalX as the expression of those genes was lost in ΔGalX on D-galactose and galactitol. We cannot exclude that GalX, XlnR and AraR are regulating \textit{ladB}. GalR was previously suggested to be uniquely present in \textit{A. nidulans} \cite{4}, but the availability of additional \textit{Aspergillus} genomes demonstrated that other members of the section nidulantes, \textit{A. versicolor} and \textit{A. sydowii}, contain an ortholog of this gene; however no orthologs were identified in any of the other sequenced \textit{Aspergillus} species (S2 Fig).

\textit{A. nidulans} does not possess an ortholog of \textit{A. niger xhrA} and therefore we propose that another, yet undescribed reductase converts L-xylo-3-hexulose in this species. A previous study of the \textit{A. niger} D-galactose oxido-reductive pathway \cite{23} described a putative reductase encoding gene (\textit{red1}), for which expression was strongly reduced (>30 fold change) in ΔgalX compared to the wild type strain on D-galactose. We identified the ortholog of this gene (AN7914) in \textit{A. nidulans} and analyzed its expression in the regulatory mutants. Similar to \textit{ladB}, the expression of the \textit{red1} gene was induced on D-galactose in the reference strain (Fig 7b). Moreover, this gene was significantly down regulated on D-galactose in the ΔaraR, ΔxlnRΔgalR and ΔaraRΔgalR double mutants and ΔΔΔ triple mutant (Fig 7b). We conclude

| Table 4. Detailed regulation of genes involved in the PCP in \textit{A. nidulans}. |
| Gene number | Gene | Transcriptional activator |
|-------------|------|--------------------------|
| AN7193      | \textit{larA} | AraR alone               |
| AN0942      | \textit{ladA} | AraR alone               |
| AN10169     | \textit{lxrA} | AraR alone               |
| AN0423      | \textit{xyrA} | AraR and XlnR, XlnR is predominate |
| AN9064      | \textit{xdhA} | AraR and XlnR, AraR is predominate |
| AN8790      | \textit{xkiA} | AraR and XlnR, AraR is predominate |

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\textit{A. nidulans galR}, \textit{xlnR} and \textit{araR}
Fig 7. Expression of orthologous genes encoding enzymes involved in the Leloir (a) and the D-galactose oxido-reductive pathway (b) in *A. niger*. The expression was measured in the reference and regulatory mutant strains of *A. nidulans* after 2h of growth on D-fructose (black bars) and D-galactose (checkered bars). The columns represent the mean and error bars represent standard deviation between biological replicates. Significant change in expression between the reference strain and the mutant was marked by the asterisk. Significant changes in expression between single and double mutants were marked by Greek symbols and braces: an alpha (α) represents a significant change between ΔA and ΔΔAΔG, a beta (β) represents a significant change between ΔG and ΔXΔG/ΔΔG, a gamma (γ) represents a significant change between ΔX and ΔXΔG.

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that AN7914 is co-regulated by XlnR, GalR and AraR in \textit{A. nidulans} and is a strong candidate to encode the not yet identified L-sorbose reductase. Further analysis, including \textit{in vitro} characterization, needs to be performed to confirm its involvement in the D-galactose oxido-reductive pathway.

In the next step of the D-galactose oxido-reductive pathway in \textit{A. niger}, D-sorbitol is converted to D-fructose by sorbitol dehydrogenase (SdhA) \cite{22}. We identified the ortholog of \textit{sdhA} (AN2666) in \textit{A. nidulans} and analyzed its expression in the regulatory mutants. The \textit{sdhA} gene was significantly down regulated on D-galactose in \textit{ΔxlnR}, \textit{ΔaraR} and double disruptants of all three regulators tested (Fig 7b). The mutation in \textit{ΔXΔA} and \textit{ΔXΔG} had the strongest effect and abolished expression of \textit{sdhA} gene (Fig 7b). Taking into account the expression levels of \textit{ladB} and \textit{sdhA} we propose that \textit{A. nidulans}, similarly to \textit{A. niger}, uses LadB and SdhA in the D-galactose oxido-reductive pathway. Both \textit{ladB} and \textit{sdhA} genes are highly induced by D-galactose and co-regulated by AraR, XlnR and GalR. Interestingly, interactions between those TFs might exceed beyond D-galactose metabolism. It was previously reported that β-galactosidase, an enzyme that removes β-linked D-galactose from polysaccharides, is induced by D-galactose (GalR/GalX) and also L-arabinose (AraR) and D-xylose (XlnR) in \textit{A. nidulans} \cite{33}.

The expression of \textit{hxkA} that encodes hexokinase involved in the last step of the oxido-reductive D-galactose catabolic pathway is not significantly reduced by any of the tested regulatory mutants (Fig 7b). Considering that the main role of \textit{hxkA} is linked to glycolysis, it is likely to have a constitutive level of expression.

It should be noted that the \textit{ΔΔΔGΔX} strain is still able to grow on galactitol (Fig 4). We have three possible explanations for that: a) AraR, GalR and XlnR are not the only regulators involved in the D-galactose oxido-reductive pathway; b) the genes remain expressed at a basal, non-regulated level; c) there is an alternative pathway involved. A previous study suggested a pathway in which L-sorbose is phosphorylated, either directly or after epimerization to D-tagatose, and subsequently cleaved into two glycolytic triose-phosphates \cite{16,17}. It should also be considered that \textit{A. nidulans} possesses two D-galactose related regulators, GalR (tested in this study) and GalX. It is possible that GalX is directly involved in regulation of other enzymes of D-galactose conversion pathways. Future studies in which double and triple mutants of GalX with AraR and XlnR are analyzed will likely reveal the role of GalX in more detail.

**Conclusions**

To summarize, the data of this study demonstrate that regulation of carbon catabolic pathways requires co-operation of two or more transcriptional factors. We have presented evidence that the D-galactose oxido-reductive pathway is co-regulated by at least three TFs in \textit{A. nidulans} (Fig 8). We have also observed a compensation effect, when in case of loss of one regulator the other takes over to maintain expression of necessary enzymes. Such a regulatory system by which metabolic pathway genes are co-regulated by multiple transcription factors especially benefits this cosmopolitan fungus that is known for its ability to quickly adapt to environmental changes. The common co-occurrence of D-galactose, L-arabinose and D-xylose in nature likely stimulated the evolution of an interactive regulatory network in which expression of genes is co-regulated by two or more TFs. It seems that this “strategy” has paid off since \textit{A. nidulans} can degrade arabinogalactan, which is a combination of D-galactose and L-arabinose, better than \textit{A. niger}. Recent study showed that \textit{A. niger} failed to grow on galactose due to the absence of inducer uptake during germination \cite{34}. In contrast, \textit{A. nidulans} as well as \textit{A. sydowi} and \textit{A. versicolor} can use D-galactose as a sole carbon source (www.fung-growth.org). GalR, which is present in these species and absent in \textit{A. niger}, may be required for galactose transporter expression during germination. Moreover, in this work we found evidence that \textit{A.
nidulans has different enzymes involved in the PCP and D-galactose pathways, like was observed for A. niger [23]. However, two of the enzymes involved in the D-galactose oxido-reductive pathway in A. nidulans differ from those of A. niger and still remain to be characterized.

Supporting Information

S1 Fig. Pairwise amino acid sequence alignment of A. nidulans XlnR-AraR (a), GalR-AraR (b) and GalR-XlnR (c). The alignment was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) [31] and visualized using Easy Sequencing in PostScript (http://esprit.ibcp.fr/ESPript/ESPript/index.php) [32]. Conserved regions are marked by shaded boxes and similar regions by unshaded boxes. The Zn$_2$Cys$_6$ binuclear DNA binding domain is underlined and the six conserved cysteine residues are indicated by stars. (PDF)

S2 Fig. Synteny of the galX region among several Aspergillus species. The galX gene is conserved while the galR gene is present only in A. nidulans, A. versicolor and A. sydowii. The positions of galX, galR, ladB and xhrA are indicated. (PDF)

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**Author Contributions**

Conceived and designed the experiments: RPDV BSG EB. Performed the experiments: JEK BSG EB AW EM. Analyzed the data: JEK EB RPDV. Wrote the paper: JEK EB RPDV.

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