

# Revisiting a ‘simple’ fungal metabolic pathway reveals redundancy, complexity and diversity

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## Summary

**Next to D-glucose, the pentoses L-arabinose and D-xylose are the main monosaccharide components of plant cell wall polysaccharides and are therefore of major importance in biotechnological applications that use plant biomass as a substrate. Pentose catabolism is one of the best-studied pathways of primary metabolism of *Aspergillus niger*, and an initial outline of this pathway with individual enzymes covering each step of the pathway has been previously established. However, although growth on L-arabinose and/or D-xylose of most pentose catabolic pathway (PCP) single deletion mutants of *A. niger* has been shown to be negatively affected, it was not abolished, suggesting the involvement of additional enzymes. Detailed analysis of the single deletion**

**mutants of the known *A. niger* PCP genes led to the identification of additional genes involved in the pathway. These results reveal a high level of complexity and redundancy in this pathway, emphasizing the need for a comprehensive understanding of metabolic pathways before entering metabolic engineering of such pathways for the generation of more efficient fungal cell factories.**

## Introduction

Substantial effort has been directed towards developing microbial platforms for production of chemicals and bio-fuels from lignocellulosic materials. In particular, filamentous fungi have received increasing interest, as they efficiently degrade plant polysaccharides and have an extensive metabolism to convert the released monomers (de Vries and Visser, 2001). This combination of extracellular enzymes to degrade polysaccharides and a complex network of metabolic pathways to convert monosaccharides into value added compounds make these fungi highly attractive cell factories.

L-arabinose and D-xylose are the most abundant monosaccharides in nature after D-glucose, being major constituents of xylan, xyloglucan and pectin (Seiboth and Metz, 2011). They are therefore highly interesting starting compounds for fungal cell factories. However, for more efficient utilization of lignocellulosic materials and increased productivity, an in-depth understanding of the pathways involved in the conversion of these sugars is required. In most fungi, L-arabinose and D-xylose are metabolized through the pentose catabolic pathway (PCP), comprised of two interconnected oxidoreductive pathway branches (Witteveen *et al.*, 1989). Both pentoses undergo oxidation, reduction and phosphorylation reactions to finally form D-xylulose-5-phosphate, which is further metabolized through the pentose phosphate pathway (PPP) (Witteveen *et al.*, 1989). For the development of fungal cell factories that convert D-xylose and/or L-arabinose to valuable compounds, manipulation of the PCP is of major importance to either accumulate desired intermediates or increase the flux through the pathway. This requires a full understanding of the pathway and its enzymes.

*Aspergillus niger* is one of the most prominent fungal cell factories used by industry. In *A. niger*, L-arabinose catabolism (Fig. 1) initiates with reduction of L-arabinose

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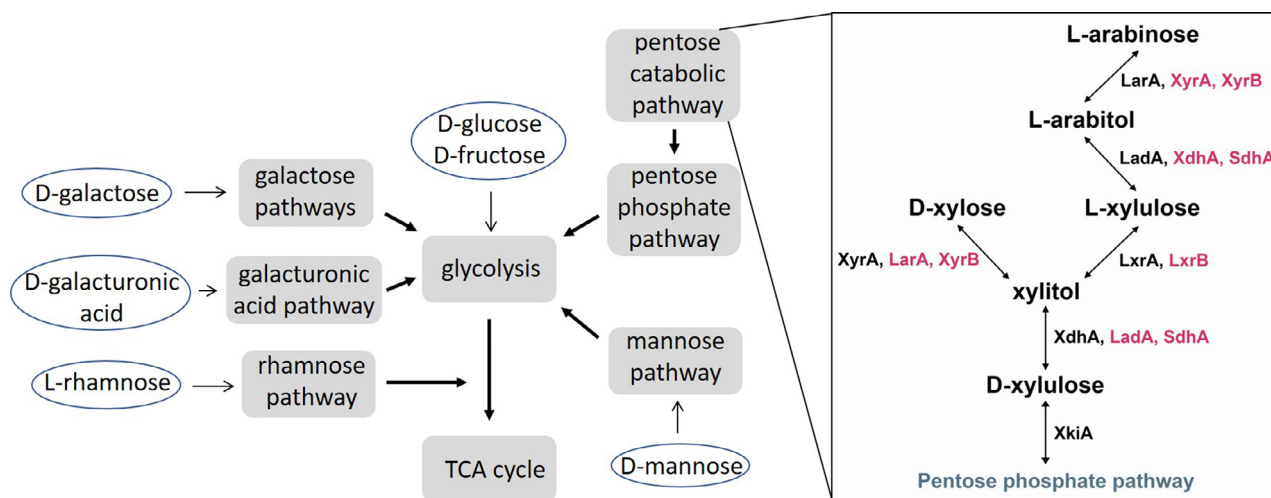
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**Fig. 1.** The links between the sugar catabolic pathways of *A. niger* and in more detail the pentose catabolic pathway (PCP). LarA = L-arabinose reductase, LadA = L-arabitol dehydrogenase, LxrA and LxrB = L-xylulose reductases, SdhA = sorbitol dehydrogenase, XyrA and XyrB = D-xylose reductases, XdhA = xylitol dehydrogenase, XkiA = D-xylulose kinase. Enzymes identified in this work to be involved in each step of the pathway are indicated in magenta.

to L-arabitol catalysed by NADPH-dependent L-arabinose reductase (LarA; EC 1.1.1.21) (Mojzita *et al.*, 2010a). L-arabitol is then converted to L-xylulose by NAD<sup>+</sup>-dependent L-arabitol dehydrogenase (LadA; EC 1.1.1.12) (de Groot *et al.*, 2007), while the resulting L-xylulose is reduced to xylitol by NADPH-dependent L-xylulose reductase (LxrA; EC 1.1.1.10) (Mojzita *et al.*, 2010b). Xylitol is the first common intermediate of the interconnected L-arabinose and D-xylose fungal catabolic pathways. D-Xylose enters the pathway at this point after its reduction to xylitol by NADPH-dependent D-xylose reductase (XyrA; EC 1.1.1.307) (Hasper *et al.*, 2000). Xylitol is further oxidized to D-xylulose by NAD<sup>+</sup>-dependent xylitol dehydrogenase (XdhA; EC 1.1.1.9) (de Groot *et al.*, 2007). In a final step, D-xylulose is converted to D-xylulose-5-phosphate by ATP-dependent D-xylulose kinase (XkiA; EC 2.7.1.17) (vanKuyk *et al.*, 2001), which can then enter the PPP.

This relatively simplistic view of the metabolic pathway with a specific enzyme for each step is not fully supported by studies in *A. niger*. While deletion of *xkiA* completely abolished growth on both pentoses (vanKuyk *et al.*, 2001), deletion of *larA* and *xyrA* only reduced growth on L-arabinose and D-xylose, respectively (Mojzita *et al.*, 2010a), and deletion of *lxrA* only slightly affected growth on L-arabinose and L-arabitol (Mojzita *et al.*, 2010b). Deletion of *xdhA* and *ladA* in the related fungus *Aspergillus oryzae* resulted in reduced rather than abolished growth (Mahmud *et al.*, 2013). These results suggest the involvement of additional enzymes in most of the PCP steps, implying that cells have evolved a higher metabolic complexity to maintain robustness. Induction of the PCP genes in *A. niger* occurs in the presence of

L-arabinose or D-xylose, mediated through the transcriptional activators AraR (Battaglia *et al.*, 2011) and XlnR (van Peij *et al.*, 1998) respectively.

Activation of XlnR is commonly believed to occur in the presence of D-xylose in filamentous fungi (van Peij *et al.*, 1998), but whether this sugar or a metabolic conversion product of it is the actual inducer has not been clearly established. In *Aspergillus tubingensis*, L-arabitol accumulation results in overactivation of XlnR (Nikolaev *et al.*, 2013). In *Trichoderma reesei*, another biotechnologically important filamentous ascomycete, L-arabitol was reported to be the inducer of its xylanolytic system (Mach-Aigner *et al.*, 2011), but a later study demonstrated that induction could occur independently through D-xylose and L-arabinose (Herold *et al.*, 2013). For AraR, it has been shown that L-arabitol, rather than L-arabinose, is the inducer in *Aspergillus nidulans* (de Vries *et al.*, 1994). The term inducer in this context refers to a compound that results in activation of a transcriptional regulator, possibly through a signal transduction pathway, and not to a compound that itself binds to the regulator.

In this study, we dissected the fungal PCP, by identifying additional genes and enzymes that participate in the different steps of the pathway, as well as their relative contribution. In addition, we constructed deletion mutants for the established (to ensure the same genetic background) and novel pathway genes in *A. niger*. Our results reveal a high level of complexity and redundancy in this pathway. This emphasizes the need for a comprehensive understanding of fungal metabolic pathways, including identification of all genes as well as the regulatory system involved, before entering metabolic

engineering of such pathways for the generation of fungal cell factories.

## Results and discussion

### Identification of additional PCP genes

We constructed all single deletion mutants of the known *A. niger* PCP genes to verify their phenotypes and their effect on the levels of the pathway intermediates and enzyme activities related to the PCP. Overall, our mutants confirmed previously reported phenotypes. Fully abolished growth only occurs for the deletion of *xkiA* ( $\Delta xkiA$ ) (Fig. 2A).

Deletion of *larA* ( $\Delta larA$ ) led to impaired growth when L-arabinose was the sole carbon source, while growth of the *xyrA* deletion mutant ( $\Delta xyrA$ ) was only minimally affected on both D-xylose and L-arabinose (Fig. 2A). Both mutants grown on D-xylose and L-arabinose showed minimal accumulation of these pentoses, as well as similar L-arabitol and xylitol levels to the reference strain (Figs 2B and 4A).

Since the ability of the  $\Delta larA$  strain to grow on L-arabinose has been attributed to the presence of the *xyrA* gene (Mojzita *et al.*, 2010a), we also constructed the double deletion mutant  $\Delta larA\Delta xyrA$ . This mutant showed a strong reduction of growth on L-arabinose and D-xylose (Fig. 2A), suggesting that both reductases are contributing, although not equally, to the conversion of these sugars. On L-arabinose, deletion of *larA* only resulted in a decrease of the L-arabinose reductase activity, while deletion of *xyrA* decreased equally L-arabinose and D-xylose reductase activity (Fig. 2C). Further decrease in L-arabinose and D-xylose reductase activities was observed for  $\Delta larA\Delta xyrA$  compared to the single deletion mutants (Fig. 2B). Both growth and enzyme activity data show that while *larA* primarily encodes for L-arabinose reductase, *xyrA* encodes for an enzyme showing similar specificity for both L-arabinose and D-xylose. In the double mutant, accumulation of L-arabinose and D-xylose was observed, while L-arabitol and xylitol accumulated at the same levels as in the reference (Fig. 4A).

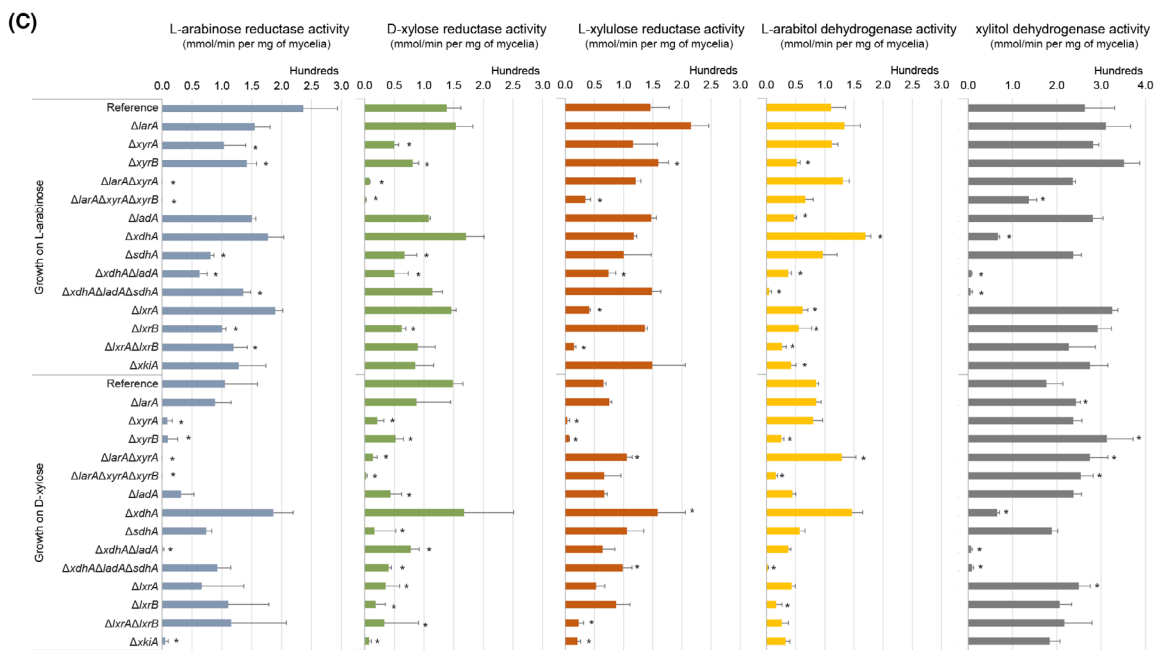
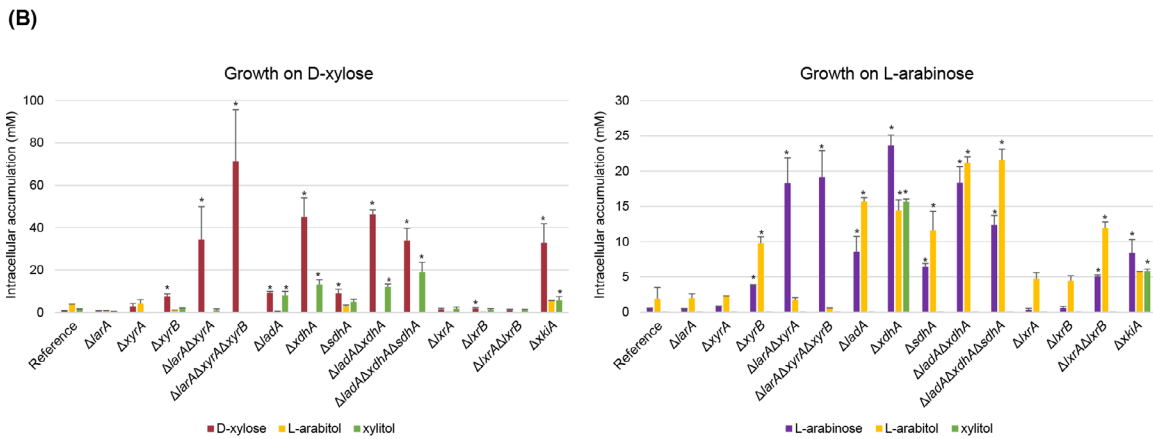
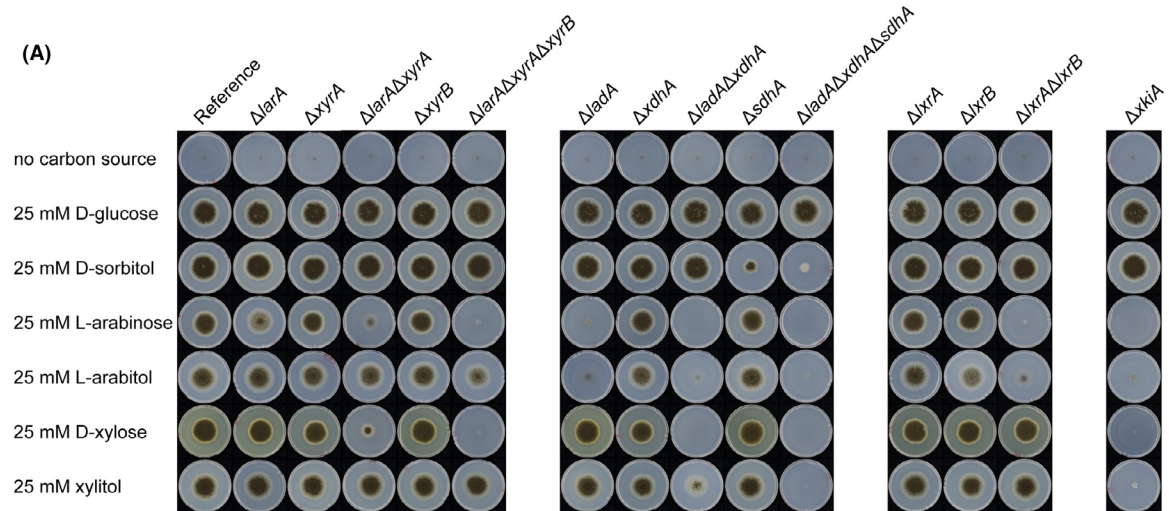
The residual growth of  $\Delta larA\Delta xyrA$  on both pentoses (Fig. 2A) indicates the involvement of additional gene(s) in the conversion of L-arabinose and D-xylose to their corresponding sugar alcohols. A previous study in *A. nidulans* aiming at identifying glycerol dehydrogenase encoding genes described an enzyme GldA that did not have glycerol dehydrogenase activity (de Vries *et al.*, 2003), but has L-arabinose and D-xylose reductase activity (A. Terebienieca, T. Chroumpi, A. Dilokpimol, M. V. Aguilar-Pontes, M. R. Mäkelä, R. P. de Vries, unpublished data). Based on data from a previous study (Gruben *et al.*, 2017), the *A. niger* homologue of *gldA*, referred to as *xyrB*, was specifically highly expressed on L-

arabinose and D-xylose, suggesting that it could be involved in the conversion of these sugars. Deletion of *xyrB* in the  $\Delta larA\Delta xyrA$  background ( $\Delta larA\Delta xyrA\Delta xyrB$ ) completely abolished growth on both pentoses (Fig. 2A), revealing that this gene can partially compensate for the loss of *larA* and *xyrA* in *A. niger*. This mutant did not accumulate any pathway intermediates (Fig. 2B), in contrast with the single and double mutants, showing that combination of all three deletions effectively blocks pentose catabolism. This is further supported by the fact that L-arabinose and D-xylose reductase activities were completely abolished in  $\Delta larA\Delta xyrA\Delta xyrB$  (Fig. 2C).

Deletion of *ladA* ( $\Delta ladA$ ) abolished growth on L-arabinose, while growth of the *xdhA* deficient mutant ( $\Delta xdhA$ ) was only slightly affected on D-xylose (Fig. 2A). Since in *T. reesei* LAD1 appears to partially compensate for XDH1 activity (Seiboth *et al.*, 2003), we also constructed the double deletion mutant  $\Delta ladA\Delta xdhA$  in order to test whether this is also applied in *A. niger*. The double deletion mutant could not grow on either pentose (Fig. 2A), confirming that LadA has also affinity for xylitol (Rutten *et al.*, 2009). When grown on L-arabinose, this mutant accumulated intracellularly more L-arabinose and L-arabitol than  $\Delta ladA$ , but similar levels of xylitol as the  $\Delta xdhA$  mutant on D-xylose (Fig. 2B).

We observed residual growth of  $\Delta ladA\Delta xdhA$  on xylitol and L-arabitol (Fig. 2A), indicating the involvement of additional enzymes in their conversions into D-xylulose and L-xylulose respectively. It was previously shown (Rutten *et al.*, 2009) that sheep liver D-sorbitol dehydrogenase has similar activity on D-sorbitol and xylitol, and significantly lower on L-arabitol. If the same is true for the *A. niger* sorbitol dehydrogenase (SdhA), which is an enzyme of the D-galactose oxidoreductive pathway (Koivistoinen *et al.*, 2012), this could explain the residual growth on xylitol and L-arabitol of the  $\Delta ladA\Delta xdhA$  mutant. Single deletion of *sdhA* ( $\Delta sdhA$ ) only reduced growth on D-sorbitol, while the phenotype of this mutant remained unaffected on all the other tested substrates compared to the reference strain (Fig. 2A). Deletion of *sdhA* in the  $\Delta ladA\Delta xdhA$  background completely abolished growth on both pentoses as well as on the pathway intermediates (Fig. 2A), confirming that SdhA compensates for the loss of LadA and XdhA in *A. niger*. In the  $\Delta ladA\Delta xdhA\Delta sdhA$  mutant, accumulation of L-arabitol on L-arabinose was slightly higher compared to the double deletion mutant  $\Delta ladA\Delta xdhA$ , while the strongest effect was in the accumulation of xylitol on D-xylose (Fig. 2B).

Finally, deletion of *lxrA* ( $\Delta lxrA$ ) did not affect growth on any of the tested substrates (Fig. 2A), in contrast to previously reported results where growth of  $\Delta lxrA$  was slightly reduced on L-arabinose (Mojzita *et al.*, 2010b). The strain in this study is from the same lineage as the



**Fig. 2.** (A) Growth profile of the *A. niger* reference strain (N593  $\Delta kusA$ ) and the PCP deletion mutants on solid MM with or without addition of carbon source. Strains were grown for 5 days at 30°C. Variation in colony diameter between replicates is < 1 mm. (B) Intracellular accumulation of intermediate metabolites of the PCP, after 2 h transfer of the mycelia to 25 mM D-xylulose or L-arabinose. (C) PCP enzyme activities (mmol/min per mg of mycelia) of the *A. niger* reference strain and the PCP deletion mutants, after 2 h transfer of the mycelia to 25 mM D-xylulose or L-arabinose. The error bars represent the standard deviation between biological replicates. Statistically significant differences from the reference strain (based on T-test,  $P < 0.05$ ) are indicated by an asterisk.

one used by us, but not identical to our strain, which may have contributed to this difference. Therefore, we also evaluated three putative L-xylulose reductase encoding genes, NRRL3\_04510 (*lxB*), NRRL3\_09880 and NRRL3\_0896, which were selected based on their expression on L-arabinose and D-xylulose in the wild type *A. niger* strain (Gruben *et al.*, 2017; Aguilar-Pontes *et al.*, 2018). Although combination of  $\Delta lxA$  with deletion of NRRL3\_09880 and NRRL3\_0896 did not affect growth on the pentoses or pathway intermediates (data not shown), growth of the  $\Delta lxA\Delta lxB$  mutant was almost abolished on L-arabinose and significantly reduced on L-arabitol (Fig. 2A), which shows that both *lxA* and *lxB* contribute to the conversion of L-arabitol to L-xylulose. Despite the absence of a phenotypic effect, the single deletion strains of *lxA* or *lxB* resulted in slightly higher accumulation of L-arabitol when grown on L-arabinose (Fig. 2B). In the double deletion mutant, the level of L-arabitol was even higher.

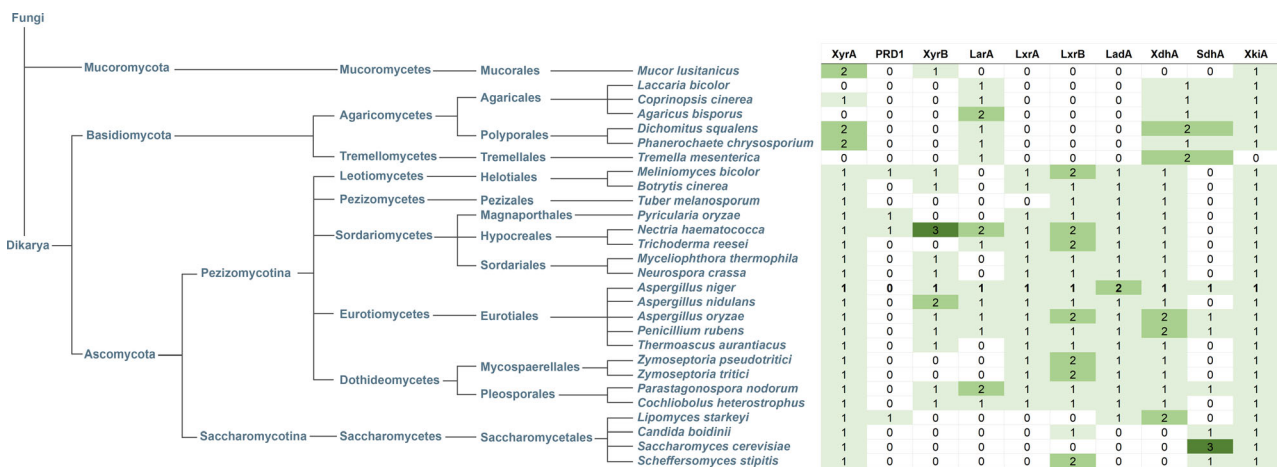
Thus, by combining phenotypic analysis, intracellular accumulation of metabolites and enzyme activities, a much higher complexity for the pentose catabolic pathway in *A. niger* was revealed, due to the involvement of several enzymes in most pathway steps. While the last enzymatic step of the pathway is catalysed by a single D-xylulose kinase, we have shown that several enzymes are involved in the other steps of the pathway, which together ensure efficient conversion of pentose sugars.

The absence of growth for the strains with multiple mutations indicates that all genes involved in those steps were identified in our study.

The only exception to this was the step from L-xylulose to xylitol. Although the double deletion  $\Delta lxA\Delta lxB$  resulted in reduced growth on L-arabinose, growth was not abolished suggesting the involvement of additional enzymes in this reaction. Deletion of NRRL3\_09880 and NRRL3\_0896 in the  $\Delta lxA\Delta lxB$  strain did not result in further growth reduction compared to  $\Delta lxA\Delta lxB$  (data not shown). Since *LxA* and *LxB* were shown to belong in different clades of the same PFAM family (PF00106), it is possible that the additional enzymes involved in this conversion could also be significantly distant from the other two, making their identification challenging.

#### Conservation of PCP genes in other fungi

Orthologues of the *A. niger* PCP genes in other fungi were identified using BlastP analysis against 28 fungal genome sequences covering the phyla Basidiomycota and Ascomycota, that together constitute the subkingdom Dikarya within the kingdom Fungi. A member of the phylum Mucoromycota, *Mucor lusitanicus*, was used as an outlier. The candidate homologues were used to construct phylogenetic trees (Fig. S1) to identify the presence or absence of orthologues in these fungi (Fig. 3).



**Fig. 3.** Presence of orthologues of the *A. niger* PCP genes in 28 fungal genome sequences covering the phyla Basidiomycota and Ascomycota. The figure is based on a BlastP analysis using the *A. niger* PCP genes as queries. The resulting hits were used in a phylogenetic analysis to identify orthologues. For the Agaricomycetes, no distinction could be made between the XdhA and SdhA orthologues. *Mucor lusitanicus* was used as an outlier.

LarA is present in most of the tested species belonging to the Agaricomycetes and Eurotiomycetes, while the presence of XyrB was largely restricted to the Pezizomycotina, but an orthologue was also detected in *M. lusitanicus*. In contrast, XyrA seems to be more uniformly present throughout the fungal kingdom, as orthologues were detected in all tested species, except for *Laccaria bicolor* and *Agaricus bisporus*, both belonging to the phylum Basidiomycota. These two species contain a LarA homologue, but are not able to grow on L-arabinose or D-xylose (www.fung-growth.org), while the Basidiomycota that contain a XyrA homologue are able to grow on these sugars. This suggests that XyrA, rather than LarA, is the main pentose reductase in basidiomycete fungi. This is not the case in other fungi, as growth on L-arabinose and D-xylose is lower in species lacking LarA and/or XyrB, showing that all three genes contribute, although not equally, to the growth of most ascomycete fungi on these sugars. This therefore mimics the results we obtained for *A. niger* and suggests an evolutionary conservation in ascomycete fungi and alteration in basidiomycete fungi. The pentose reductase PRD1, a paralog of XyrA, is involved in pentose catabolism in *Pyricularia oryzae* (Klaubauf *et al.*, 2013) and is present in a few other Sordariomycetes. However, PRD1 homologues are absent in *A. niger* and other Eurotiomycetes. In our study, PRD1 homologues were found only in four tested ascomycete fungi (*P. oryzae*, *Nectria haematococca*, *Melionomyces bicolor* and *Lipomyces starkeyi*), which also all contained a XyrA homologue.

BlastP analysis and phylogeny of LxrA and LxrB revealed candidate homologues for both of them in all 16 analysed species of the Pezizomycotina, but not in the Basidiomycota and *M. lusitanicus*. LxrA homologues were also absent in the Saccharomycotina. Similar to LxrA and LxrB, LadA and XdhA were conserved in all analysed fungi of the Pezizomycotina. However, SdhA was only present in some Eurotiomycete species. In Basidiomycota, the absence of LadA, LxrA and LxrB can explain the limited growth of these species on L-arabinose (www.fung-growth.org), while the presence of a novel XdhA/SdhA group in this fungal phylum could explain their growth on D-xylose.

Finally, BlastP analysis of the D-xylulose kinase XkiA identified homologues in all analysed species, except for *Tremella mesenterica*, which belongs to the class Tremellomycetes of the Basidiomycota. This fungus lacks most PCP genes, which may suggest the absence of the PCP or use of different enzymes or pathways for the catabolism of pentoses. As no growth data are available for this species, its ability to use these sugars as a carbon source cannot be validated at this point.

Our homology and phylogeny analysis revealed that the PCP is not highly conserved across the fungal kingdom.

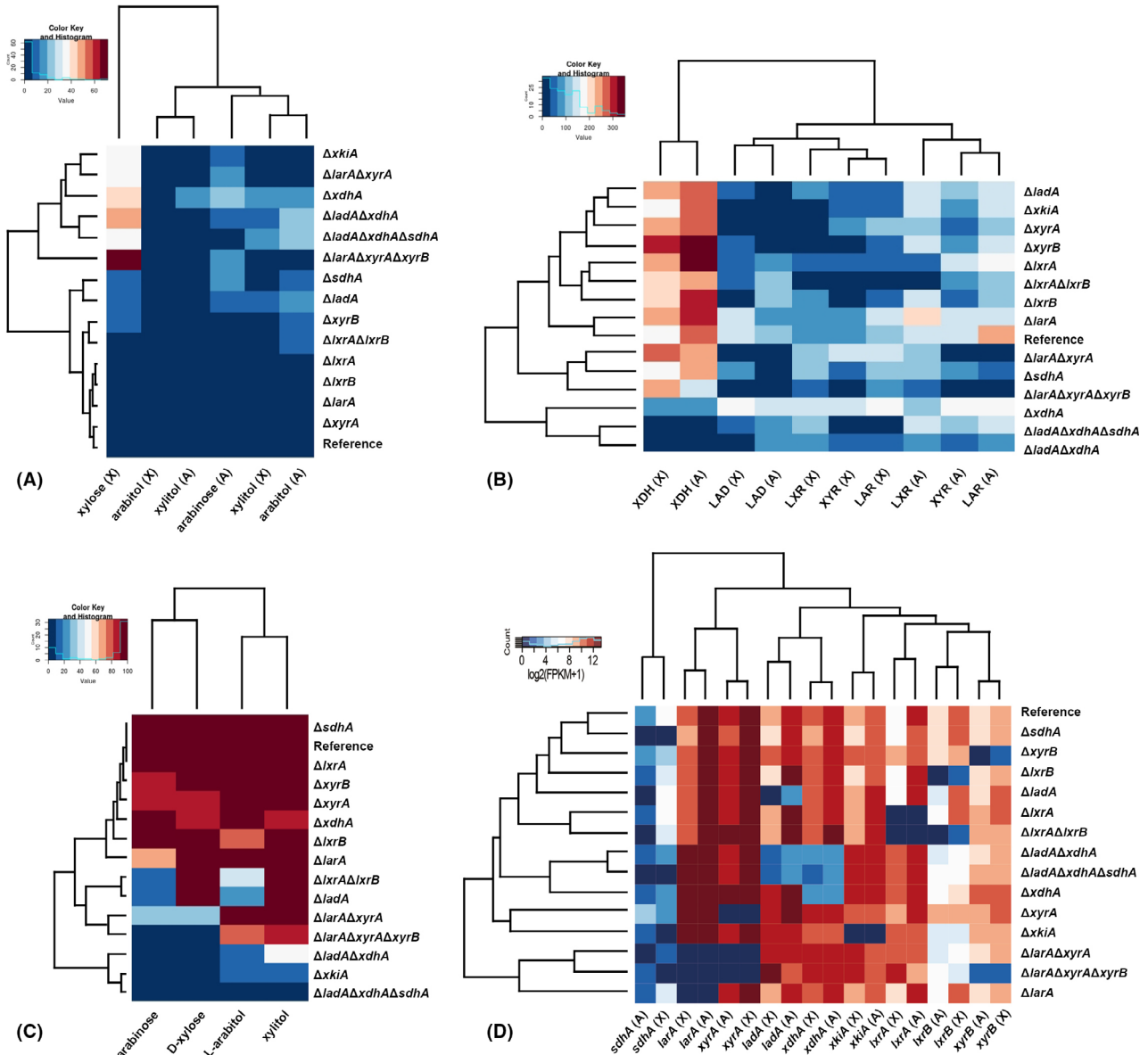
The presence of all PCP genes in the Pezizomycotina results in efficient utilization of both L-arabinose and D-xylose for growth, while in the Saccharomycotina, the lack of most of the PCP genes clearly affects growth on these carbon sources. In particular, the absence of XdhA results in inability of these fungi to grow on D-xylose, while the absence of LarA, LadA and LxrA and LxrB explain their inability to grow on L-arabinose. Recently, a positive correlation was found for subset of Aspergilli between the presence of a gene predicted to encode a catabolic enzyme and their ability to grow on a specific carbon source (de Vries *et al.*, 2017). While *M. lusitanicus* lacks most orthologues of the *A. niger* PCP genes, it grows efficiently on both sugars, suggesting the involvement of other enzymes or pathways in pentose catabolism in this fungus. The larger phylogenetic distance of Mucoromycota from the rest of the studied fungi may explain the different evolution of pentose catabolism.

These results further exemplify the need for detailed analysis of metabolic pathways in specific fungi, before these fungi can, e.g., be efficiently used in metabolic engineering strategies for biotechnology. Direct extrapolation of the available knowledge on the metabolism from one fungus to the other can be misleading, since the conservation even of this 'simple' pathway across the fungal kingdom is not ubiquitous.

#### *Effect of the metabolic deletion mutants on the PCP*

The multi-level analysis performed in this study provides an opportunity to evaluate whether the effects in the metabolic mutants are consistent across all the studied levels (gene expression, enzyme activity, accumulation of intermediates and growth on PCP compounds). However, different hierarchical clustering of the strains was observed for each of these levels (Fig. 4).

Accumulation of xylitol in all the dehydrogenase mutants on D-xylose shows that LadA, XdhA and SdhA are all involved in the conversion of xylitol to D-xylulose (Fig. 4A). The highest xylitol accumulation in  $\Delta xdhA$  points towards the role of XdhA as the main xylitol dehydrogenase (XDH) in *A. niger*, while LadA and SdhA also contribute to this activity. This is also confirmed by the enzyme activity data, where all the strains showing low XDH activity lacked *xdhA* (Fig. 4B). However, the reduction in XDH activity was not as strong in the  $\Delta xdhA$  mutant as in the double or the triple dehydrogenase mutants, clearly indicating the involvement of the other two enzymes in this metabolic step. On D-xylose, we noticed higher L-arabitol dehydrogenase (LAD) activity and expression of *ladA* in the  $\Delta xdhA$  mutant compared to the reference (Fig. 4B and D respectively). This is probably because the fungus is trying to compensate for the loss of *xdhA* by increasing the expression of *ladA* and as a result



**Fig. 4.** Hierarchical clustering of (A) the intracellular concentration (mM) of PCP intermediate metabolites, (B) PCP enzyme activities (mmol/min per mg of mycelia), (C) growth on pentose sugars and polyols (the colour code displayed represents the difference in colony diameter) and (D) the expression profiles (the colour code displayed represents averaged and logged expression values (FPKM + 1) of triplicates) of genes linked to pentose catabolism in the *A. niger* reference strain and PCP mutants, after 2 h transfer of the mycelia to 25 mM L-arabinose (A) or D-xylose (X). No significant differences were observed for the expression profile of the strains on D-glucose (data not shown). LAR = L-arabinose reductase, LAD = L-arabitol dehydrogenase, LXR = L-xylulose reductase, XYR = D-xylose reductase, XDH = xylitol dehydrogenase.

LAD activity. This could also be the reason why the  $\Delta xdhA$  mutant only had a small growth reduction on D-xylose (Fig. 4C). Similarly, although to a smaller extent, deletion of *ladA* resulted in slightly improved XDH activity and expression of *xdhA* on D-xylose (Fig. 4B and D respectively), which could also explain the almost unaffected phenotype of  $\Delta ladA$  on D-xylose (Fig. 4C).

On L-arabinose, deletion of *xdhA* caused accumulation of both L-arabitol and xylitol (Fig. 4A). However, in both

$\Delta ladA\Delta xdhA$  and  $\Delta ladA\Delta xdhA\Delta sdhA$ , accumulation of xylitol did no longer occur on L-arabinose, confirming that LadA is the most important enzyme for conversion of L-arabitol to L-xylulose. By deleting *ladA*, we can effectively prevent most of the conversion towards xylitol. The increased accumulation of L-arabitol in the double and triple deletion dehydrogenase mutants (Fig. 4A) confirms the complementary role of XdhA and SdhA in this conversion (Fig. 4C). The LAD activity on L-arabinose and

D-xylose does also correlate with the metabolite and growth data for these mutants. Knocking out *xdhA* did not have as large an effect, while deletion of *ladA* had a strong effect in LAD activity (Fig. 4B). Double deletion of *ladA* and *xdhA* only had a small difference with  $\Delta ladA$ , but additional deletion of *sdhA* contributed to a further reduction of LAD activity. This indicates that *sdhA* plays a stronger role in LAD activity compared to *xdhA*.

According to the phenotype of the L-arabinose/D-xylose reductase mutants (Fig. 4C), LarA appears to be the most dominant enzyme in the conversion of L-arabinose to L-arabitol, while D-xylose conversion requires the involvement of XyrA, LarA and XyrB. However, although *larA* deletion resulted in growth reduction on L-arabinose (Fig. 4C), intracellular accumulation of this pentose sugar was not observed in  $\Delta larA$  (Fig. 4A). The highest accumulation of pentoses occurred in the  $\Delta xyrB$ , showing the importance of XyrB to conversion of metabolites (Fig. 4A), irrespective of the lack of phenotypic effect of this mutant on both pentoses. As expected, double and triple mutants,  $\Delta larA\Delta xyrA$  and  $\Delta larA\Delta xyrA\Delta xyrB$ , accumulated high amounts of L-arabinose and D-xylose when grown on these sugars.

In contrast to the dehydrogenases, D-xylose reductase (XYR) and L-arabinose reductase (LAR) activities clustered according to the carbon source and not according to the different enzyme activity (Fig. 4B). This shows that the activities of XYR and LAR overlap more strongly than those of XDH and LAD. For all the pentose reductase mutants, except for  $\Delta larA$ , the LAR and XYR activities on D-xylose were reduced compared to the reference, while on L-arabinose these activities significantly dropped only in the multiple deletion mutants  $\Delta larA\Delta xyrA$  and  $\Delta larA\Delta xyrA\Delta xyrB$ . Reduced LAR and XYR activities were also observed in most of the dehydrogenase mutants (Fig. 4B), which was surprising since these mutants accumulate L-arabitol on L-arabinose (Fig. 4A). If we assume that *larA* is regulated by the L-arabitol induced AraR, the reduced activity in these mutants should be attributed to XyrA and XyrB rather than LarA. Deletion of either *larA*, *xyrA* or *xyrB* did not result in upregulation of the remaining L-arabinose/D-xylose reductase genes (Fig. 4D). Therefore, the almost unaffected phenotype of these single mutants on both pentoses cannot be explained by increased activity of the remaining enzymes (Fig. 4C). Deletion of *xyrA*, *xyrB* and *xdhA* resulted in upregulation of the AraR responsive *ladA*, *lxrA* and *xkiA* genes on D-xylose (Fig. 4D), although only accumulation of D-xylose was observed under these conditions (Fig. 4A).

On L-arabinose, single deletions of *lxrA* and *lxrB* did not result in accumulation of L-arabinose and L-arabitol (Fig. 4A), but the double deletion resulted in accumulation of both intermediates, confirming the growth data of these

mutants (Fig. 4C). As expected, higher L-xylose reductase (LXR) activity was observed on L-arabinose than D-xylose (Fig. 4B). On L-arabinose, strong reduction of LXR activity was observed after deletion of *lxrA*, while deletion of *lxrB* did not have a significant effect. Double deletion of *lxrA* and *lxrB* resulted in even stronger reduction compared to the single *lxrA* deletion mutant. However, residual LXR activity was still present in  $\Delta lxrA\Delta lxrB$  mutant (Fig. 2C), which shows again that additional enzyme(s) are involved in this activity.

Combination of the metabolite and expression data (Fig. 4A and D) revealed that for the strains with intracellular accumulation of D-xylose (Fig. 4A), the expression of *xyrA*, which is regulated by the D-xylose induced XInR regulator, is not higher than the reference (Fig. 4D). This suggests that accumulation of D-xylose does not result in higher activation of XInR. Similarly, in the dehydrogenase mutants where L-arabitol accumulated on L-arabinose (Fig. 4A), the expression of the L-arabitol induced AraR responsive genes was also not upregulated compared to the reference (Fig. 4D), showing again that accumulation of L-arabitol does not lead to higher AraR-mediated expression.

All the above results show that despite the fact that multiple deletions are needed to have a strong phenotypic effect (Fig. 4C), deletion of a single gene can strongly reduce the overall enzyme activity of one catalytic step (Fig. 4B). It is possible that the different cultivation methods used for growth and enzyme assays (solid and liquid cultivation respectively) could contribute to this observation, as differences in aeration due to agitation and nutrient availability may affect the physiology of the fungus. Another possible explanation is that the activities of these enzymes in the reference strain during growth on monosaccharides are present at higher levels than is needed to maintain normal growth. Therefore, judging the importance of one gene for the pathway based exclusively on the expression level or the activity level of the corresponding enzyme could be misleading, because it could be that at least on pure sugars several genes are expressed and the enzymes are produced in higher amounts than actually required. Probably on crude plant biomass substrates, where these sugars are at more natural levels, the importance of these mutations may be drastically different. On these substrates, additional enzymes may be produced that have side activity on the PCP sugars and therefore contribute to the conversion of D-xylose or L-arabinose.

#### *Genetic engineering of the PCP affects not only pentose release and conversion, but also other biological functions*

Gene Ontology (GO) enrichment analysis of the expression data of the metabolic mutants revealed not only a



broad effect of these mutations, but also significant differences in the effect of the mutations during growth of *A. niger* on D-xylose and L-arabinose respectively (Fig. S2). During growth on D-xylose, the expression of genes in many GO terms was elevated in the mutants, especially in those in which multiple genes encoding pentose reductases or polyol dehydrogenases are deleted (Fig. S2a). In contrast, on L-arabinose, the expression of genes included in many GO terms was reduced in several mutants (Fig. S2b). The overall effect on the expression pattern was relatively small in most single mutants, especially in  $\Delta larA$ ,  $\Delta xyxB$ ,  $\Delta lxrA$  and  $\Delta lxrB$ . Most of the affected GO terms were involved in carbon metabolism, cell cycle or nitrogen metabolism.

Considering the importance of the PCP and the associated regulators, AraR and XlnR, for plant biomass conversion, we analysed in more detail the expression of the Carbohydrate-Active Enzyme (CAZy) genes related to plant biomass degradation. We compared the expression profile of the PCP genes and confirmed sugar transporters and the plant biomass degradation-related CAZy genes to identify patterns of co-expression. Surprisingly, some of the PCP genes did not cluster with the other confirmed AraR- and XlnR-regulated genes (Gruben *et al.*, 2017). A subset of genes, i.e. *xyrB*, *sdhA*, *xyrA* and *lxrB*, were almost completely separate from the CAZy genes in the heatmap (Fig. S3), indicating that they behave very differently compared to the other XlnR- and AraR-regulated genes. Interestingly, this set contains all the novel identified PCP genes (*xyrB*, *lxrB*, *sdhA*), which may explain why it was more difficult to identify their involvement in the PCP, compared to the other pathway genes.

Of all identified *A. niger* plant cell wall degradation-related CAZy genes, those regulated by XlnR and/or AraR were most significantly expressed as well as most strongly affected in the PCP mutants on both sugars (Table S1). However, some other CAZy genes that have not been shown to be under the control of either XlnR or AraR were also expressed and upregulated in the mutants under these conditions. For example, on D-xylose, both NRRL3\_742 and NRRL3\_2524 (*agdB*) followed the same expression profile as several AraR- and/or XlnR-regulated genes (Table S1a), which shows that these genes are probably also under the control of these regulators. Compared to the reference strain, all affected genes on D-xylose were particularly upregulated in the multiple pentose reductase mutants, in the polyol dehydrogenase mutants especially where *xdhA* was missing, and in the  $\Delta xkiA$  mutant. In these mutants, intracellular accumulation of D-xylose was observed (Fig. 4A), which has been previously identified as the inducer of the XlnR transcriptional regulator (Table S1b). On L-arabinose, several other CAZy genes had the same expression profile as the already identified

AraR- and/or XlnR-regulated genes. The XlnR-regulated genes were more affected in mutants that particularly accumulated L-arabinose, while the expression of the AraR-regulated genes was increased in the mutants accumulating both L-arabinose and L-arabitol. In the mutants with low accumulation of L-arabinose and L-arabitol (< 5 mM), such as  $\Delta larA$ ,  $\Delta xyrA$ ,  $\Delta lxrA$  and  $\Delta lxrB$  (Fig. 2B), we did not observe significant differences in CAZy expression profile compared to the reference strain.

In general, we could not observe a clear distinction between AraR- and XlnR-regulated genes, probably because the metabolic steps affect both regulators. Several of the PCP and CAZy genes are under control of both AraR and XlnR, which further complicates the expression profiles.

Also, there is no clear correlation between the level of intermediates in the different strains and the expression profiles of the PCP or the other AraR- and XlnR-regulated genes. The two regulators together control the pathway, which means that if the flux through the pathway is changed, it affects both regulators, and as a result all the target genes. Interestingly, the strains that result in similar accumulation of intermediates do not necessarily result in similar expression patterns, while the concentration of the inducer also does not seem to be related to the level of the induction. Neither accumulation of D-xylose results in higher expression of the XlnR-regulated genes nor accumulation of L-arabitol in higher expression of the AraR-regulated genes compared to the reference strain. These results suggest that maximal induction of XlnR- or AraR-mediated PCP gene expression already occurs in the reference strain (Fig. 4D), and therefore, accumulation of PCP intermediates does not have an additional effect.

This observation confirms a previous study (de Vries *et al.*, 1999) on a small number of XlnR-regulated xylanolytic genes, where concentration-independent induction was suggested. Specifically, it was shown that the induction of these genes was already high in low concentrations of the inducer D-xylose, and higher D-xylose concentrations did not result in increased expression. In addition, their results clearly indicate that D-xylose concentrations higher than 1 mM lead in repression of the expression of these xylanolytic genes mediated by the carbon catabolite repressor protein CreA. This observation could explain why in our strains, where the balance of intermediate metabolites has completely changed, the induction pattern does not change. In strains where the intermediates of the pathway accumulated, much higher expression was not automatically observed, which may be due to repression by the high concentration of L-arabinose and D-xylose. As a consequence, increased accumulation of intermediates could result in reduction rather than induction of the related genes.

## Conclusion

Fungal cell factories are receiving increasing interest in the biobased economy to replace traditional chemical processes. However, engineering of fungal metabolism aiming at fine-tuning of CAZyme production or redirecting pathways towards accumulation of valuable products can be very challenging, even for relatively simple pathways, such as the PCP, and well-studied fungi, such as *A. niger*. In our study, we demonstrated a much higher complexity of the PCP, involving multiple enzymes in most steps of the pathway, which should all be taken into account when engineering the pathway towards the accumulation of a specific intermediate, such as xylitol. This emphasizes the importance of a deeper understanding of metabolic pathways, including identification of all genes as well as the regulatory system involved, to improve the efficiency of metabolic engineering aiming at designing better and more versatile fungal cell.

## Experimental procedures

### *Strains, media and growth conditions*

All *A. niger* strains described in this study were deposited in the CBS strain collection of the Westerdijk Fungal Biodiversity Institute under accession numbers listed in Table S2a. All strains were grown at 30°C using Minimal Medium (MM, pH 6) or Complete Medium (CM, pH 6) (de Vries *et al.*, 2004) with the appropriate carbon source. For solid cultivation, 1.5% (w/v) agar was added in the medium. Media of auxotrophic strains were supplemented with 1.22 g l<sup>-1</sup> uridine, while a final concentration of 1.3 mg ml<sup>-1</sup> of 5-fluoroorotic acid (5-FOA) (Nødvig *et al.*, 2015) was used for counterselection of strains carrying the *pyrG* marker gene on the self-replicating plasmid.

Spores were harvested from CM agar plates in ACES buffer (10 mM N-(2-acetamido)-2-amino-ethanesulfonic acid, 0.02% Tween 80, pH 6.8), after five days of growth, and counted using a haemocytometer. Growth profiling plates were inoculated with 1000 spores in 2 µl ACES buffer and incubated at 30°C for 5 days. All liquid cultures were incubated in an orbital shaker at 250 r.p.m. and 30°C. For transfer experiments, 250 ml CM with 2% D-fructose in 1 l Erlenmeyer flasks were inoculated with 10<sup>6</sup> spores ml<sup>-1</sup> and incubated for 16 h. Thereafter, the mycelia were harvested by filtration on sterile cheesecloth and washed with MM and ~0.5 g (dry weight) was transferred to 250 ml Erlenmeyer flasks containing 50 ml MM with 25 mM D-glucose, L-arabinose or D-xylose. 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. Samples were stored at -80°C.

### *Identification of candidate genes*

Genes included in this study were annotated in the manually curated *A. niger* NRRL 3 metabolic network-based orthology, phylogenomics and *A. niger* NRRL 3 functional annotation (Aguilar-Pontes *et al.*, 2018; Vesth *et al.*, 2018).

NRRL3\_10868 (*xyrB*) encoding a putative D-xylose/L-arabinose reductase in *A. niger* was identified as homologue to the *A. nidulans glcA* gene (de Vries *et al.*, 2003). NRRL3\_04510 (*lxrB*), NRRL3\_09880 and NRRL3\_0896 were selected among the annotated reductases based on in-house expression data.

### *Protoplast-mediated transformation, mutant purification and screening*

For the construction of all the mutants described in this study, we used the CRISPR/Cas9 system (Song *et al.*, 2018). Geneious R11 software was used to identify 20 bp guide sequences from the *A. niger* NRRL 3 genome, (Kearse *et al.*, 2012). The guide sequences and plasmids used in this study are listed in Table S2b.

To construct linear deletion DNA cassettes, we amplified the upstream and downstream flanking regions of the genes by PCR using gene specific primers (Table S2c). We performed PCR amplification using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Nieuwegein, the Netherlands), following manufacturer's instructions, and using genomic DNA from the reference strain as a template. The upstream reverse and the downstream forward primers were designed to harbour a barcode sequence [actgctaggattcgctatcg]. This sequence was used as the homologous region for the fusion of these two fragments in a PCR reaction, to generate the linear deletion DNA cassette. The amplified deletion cassettes were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Hoevelaken, the Netherlands).

*Aspergillus niger* protoplasting was performed as previously described (Chroumpi *et al.*, 2020). Polyethylene glycol (PEG)-mediated transformation of *A. niger* protoplasts was performed as described in detail by (Kowalczyk *et al.*, 2017). All transformations were carried out using 0.8 µg of ANEp8-Cas9-gRNA plasmid DNA together with 4–6 µg of purified linear deletion DNA cassette. Five colonies per mutant were randomly selected from the transformation plates and streak-purified twice on MM plates. For *A. niger* colony PCR, genomic template DNA was isolated from mycelia of putative deletion strains using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA). Correct mutants were identified by PCR amplification of the sequences flanking the CRISPR/Cas9 cut site, using primers listed in

Table S2c. Prior to storage, mutants were re-inoculated twice on MM plates supplemented with 1% D-glucose and uridine, and subsequently on plates with 5-FOA (Nødvig *et al.*, 2015) to remove the self-replicating plasmid.

### Sequence analysis

Amino acid sequences were retrieved from MycoCosm (Grigoriev *et al.*, 2013) (<https://mycocosm.jgi.doe.gov/mycocosm/home>) and the *Aspergillus* Genome database ([www.aspgd.org](http://www.aspgd.org)). The amino acid sequences of the *A. niger* PCP enzymes (Aguilar-Pontes *et al.*, 2018), *Magnaporthe oryzae* pentose reductase PRD1 (Klaubauf *et al.*, 2013) and *Aspergillus nidulans* glycerol dehydrogenase GldB (de Vries *et al.*, 2003) were used as a query for BlastP analysis to identify the orthologues in the other fungi. Sequences were aligned using MAFFT (Kato *et al.*, 2009) and manually corrected. A phylogenetic tree was reconstructed using MEGA X software (Tamura *et al.*, 2013) with the maximum likelihood algorithm, using 500 bootstraps with using the glycerol dehydrogenase (GldB) group as an outgroup. The maximum likelihood tree was then displayed with bootstrap values at the nodes if their support was at least 50%.

### Transcriptome sequencing and analysis

The transcriptomic response of the PCP deletion mutants induced after 2 h on D-glucose, L-arabinose or D-xylose was analysed using RNA-sequencing. Total RNA was extracted from ground mycelial samples using TRIzol<sup>®</sup> reagent (Invitrogen, Merelbeke, Belgium) and purified with the NucleoSpin<sup>®</sup> RNA Clean-up Kit (Macherey-Nagel, Düren, Germany), while contaminant gDNA was removed by rDNase treatment directly on the silica membrane. The RNA quality and quantity were analysed with a RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies, Middelburg, The Netherlands). Purification of mRNA, synthesis of cDNA library and sequencing were conducted at DOE Joint Genome Institute (JGI) as described previously (Chroumpi *et al.*, 2020).

The reads from all RNAseq samples were deposited with the Sequence Read Archive at NCBI with sample accession numbers SRP225864–SRP225877, SRP242739–SRP242757, SRP242763–SRP242774, SRP242787–SRP242846, SRP242859–SRP242894, SRP242906–SRP242928 and SRP242942–SRP242953. 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  $P_{adj} < 0.01$ .

The Gene Ontology (GO) annotation was retrieved from JGI MycoCosm database ([https://genome.jgi.doe.gov/Aspni\\_NRR13\\_1/Aspni\\_NRR13\\_1.home.html](https://genome.jgi.doe.gov/Aspni_NRR13_1/Aspni_NRR13_1.home.html)), and

the GO annotation database from R Bioconductor was used to map their ancestor nodes in the GO hierarchy. The GO biological process terms enriched within the significant differentially expressed gene lists compared to the genome background were detected by a hypergeometric distribution model calculated with in-house script. The P-values for multiple tests were corrected with Benjamini and Hochberg's method, and significantly enriched GO terms were selected with  $P$ -values  $< 0.01$ .

### Preparation of *A. niger* cell-free extracts

Frozen ground mycelial samples were resuspended in cold 1 ml extraction buffer (50 mM K-phosphate, pH 7.0, 5 mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol and 0.5 mM EDTA) and carefully mixed by pipetting. The mixture was then centrifuged (10 min at 14 000 r.p.m., 4°C), and the supernatant was collected for further analysis (i.e. enzymatic activity assays and determination of intracellular concentrations of sugars and sugar alcohols).

### Enzyme activity assays

All enzyme activities were measured in the cell-free extracts of the PCP mutants after 2 h transfer of the mycelia to 25 mM D-glucose, L-arabinose or D-xylose. Two technical replicates were performed on biological triplicates, and these were averaged in the graphs. Enzyme activities were normalized based on the total protein content of the cell-free extracts, which was measured using the Pierce<sup>™</sup> BCA Protein assay kit (Thermo Fisher Scientific).

Reductase activities were measured by detection of NADPH depletion, using 50 mM Na-phosphate buffer, pH 7.0, 0.2 mM NADPH and 100 mM L-arabinose and D-xylose or 10 mM L-xylulose (Biosynth Carbosynth, MX07932). Dehydrogenase activities were determined by detection of NAD<sup>+</sup> depletion, using 100 mM glycine/NaOH buffer, pH 9.6, 2.5 mM NAD<sup>+</sup> and 100 mM L-arabitol and xylitol. For both reductase and dehydrogenase assays, 40 µl of cell-free extracts was used per reaction in a final volume of 200 µl. Absorbance changes for NADPH and NAD<sup>+</sup> at 340 nm ( $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ) were monitored spectrophotometrically at 30°C in clear flat-bottom 96-well plates, using a plate reader FLUOstar<sup>®</sup> Optima (BMG Labtech, Ortenberg, Germany).

### Determination of sugars and sugar alcohols

The intracellular and extracellular concentrations of L-arabinose, D-xylose, L-arabitol and xylitol were determined by using high-performance anion-exchange chromatography (HPAEC, Thermo) as previously described

(Mäkelä *et al.*, 2016). Quantification was performed by external standard calibration. Reference sugars and sugar alcohols were used from 2.5 to 200  $\mu$ M. Samples were diluted 100-fold in water before HPLC analysis.

#### Statistical analysis

A two-tailed distribution *t*-test was conducted to compare the reference strain to the metabolic deletion mutant strains grown on D-xylose and L-arabinose. Based on three biological replicates for each dataset, the *t*-test was evaluated with Microsoft Excel and parameters included the two-sample equal variance. Further, the *t*-test score was expressed as *P*-value with the main assumption that if the value is < 0.05 the datasets are significantly different (marked in Fig. 2 with an asterisk \*).

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#### Conflict of interests

The authors declare no conflict of interests.

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### Supporting information

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

**Fig. S1.** Phylogenetic analysis of (a) L-arabinose/D-xylulose reductases, (b) D-xylulose kinase, (c) L-arabitol/xylitol dehydrogenases and (d) L-xylulose reductases. The tree is a representative Maximum Likelihood tree and bootstrap values (> 50) supported by the Maximum Likelihood algorithm are indicated on the nodes. LarA = L-arabinose reductase, XyrA and XyrB = D-xylulose reductases, PRD1 = pentose reductase, YPR1/GCY1 = yeast glycerol dehydrogenases, LxrA and LxrB = L-xylulose reductases, XkiA = D-xylulose kinase, LadA = L-arabitol dehydrogenase, LadB = galactitol dehydrogenase, LadC = enzyme with homology to LadA and LadB, SdhA = sorbitol dehydrogenase, XdhA = xylitol dehydrogenase, GldB = filamentous fungal glycerol dehydrogenase, GlkA = glucokinase, HxkA = hexokinase. Colours reflect the taxonomic group of the fungi. The *A. niger* enzymes used as queries are depicted in bold.

**Fig. S2.** Gene Ontology (GO) terms associated with the function of genes upregulated during growth on (a) D-xylulose and (b) L-arabinose in the *A. niger* reference strain (N593  $\Delta kusA$ ) and the PCP deletion mutants. The size and colour of the circles represent the number of genes and statistical significance of enriched GO terms, respectively.

**Fig. S3.** Hierarchical clustering of the PCP and the AraR (blue), XlnR (magenta) and AraR/XlnR (green) regulated CAZy genes with significantly upregulated expression levels in the *A. niger* reference strain (N593  $\Delta kusA$ ) and the PCP deletion mutants, after 2 h transfer of the mycelia to 25 mM L-arabinose (A) or D-xylulose (X). The colour code displayed represents averaged and logged expression values (FPKM + 1) of triplicates. Gene names are expressed as NRRL3\_gene ID|gene name|CAZy family|regulon.

**Table S1.** Significantly upregulated genes encoding CAZymes in *A. niger* PCP deletion mutants, after 2 h transfer of the mycelia to 25 mM D-xylulose or L-arabinose.

**Table S2.** *A. niger* strains, plasmids and primers used in this study.