

A Novel L-Xylulose Reductase Essential for L-Arabinose Catabolism in *Trichoderma reesei*

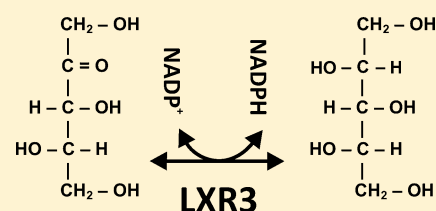
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S Supporting Information

ABSTRACT: L-Xylulose reductases belong to the superfamily of short chain dehydrogenases and reductases (SDRs) and catalyze the NAD(P)H-dependent reduction of L-xylulose to xylitol in L-arabinose and glucuronic acid catabolism. Here we report the identification of a novel L-xylulose reductase LXR3 in the fungus *Trichoderma reesei* by a bioinformatic approach in combination with a functional analysis. LXR3, a 31 kDa protein, catalyzes the reduction of L-xylulose to xylitol via NADPH and is also able to convert D-xylulose, D-ribulose, L-sorbose, and D-fructose to their corresponding polyols. Transcription of *lxr3* is specifically induced by L-arabinose and L-arabitol. Deletion of *lxr3* affects growth on L-arabinose and L-arabitol and reduces total NADPH-dependent LXR activity in cell free extracts. A phylogenetic analysis of known L-xylulose reductases shows that LXR3 is phylogenetically different from the *Aspergillus niger* L-xylulose reductase LxrA and, moreover, that all identified true L-xylulose reductases belong to different clades within the superfamily of SDRs. This indicates that the enzymes responsible for the reduction of L-xylulose in L-arabinose and glucuronic acid catabolic pathways have evolved independently and that even the fungal LXRs of the L-arabinose catabolic pathway have evolved in different clades of the superfamily of SDRs.



Plant cell walls consist of the polysaccharides cellulose, different hemicelluloses, and pectins and the complex polymer lignin. While cellulose is a linear β -1,4-linked D-glucose polymer, the structure and composition of hemicelluloses and pectins are more diverse. Following D-xylose, L-arabinose is the second most abundant pentose in hemicelluloses and pectins. It is present as a single residue or a short side chain in arabinoxylans or as larger branched side chains in the form of arabinan or arabinogalactan in pectins.^{1,2} Though the enzymatic steps for the catabolism of L-arabinose were described in the 1960s,³ most of the genes encoding the enzymes of this five-step pathway were only characterized recently.⁴ One reason for this might be that an L-arabinose pathway is not found in the fungal model organism *Saccharomyces cerevisiae*. Recently, considerable efforts were undertaken to fully elucidate this pathway for introduction of such a trait into *S. cerevisiae* to allow a complete conversion of plant biomass to, e.g., advanced biofuels or other biorefinery products.^{5,6} Degradation of L-arabinose in fungi usually consists of four oxidoreductive reactions and a final phosphorylation step, distinguishing this path from the different pathways for bacterial L-arabinose catabolism. The last two reactions of the fungal L-arabinose pathway are shared with the D-xylose catabolic pathway (Figure 1). The bacterial isomerase pathway consists of an L-arabinose isomerase, ribulokinase, and L-ribulose phosphate-4-epimerase, while the enzyme sequence of the oxidative pathway consists of L-arabinose dehydrogenase, L-arabinolactonase, L-arabonate dehydratase, L-2-keto-3-deoxy-arabonate dehydratase, and 2,5-

dioxovalerate dehydrogenase, the end product being α -ketoglutarate. In a modification of this oxidative pathway, L-2-keto-3-deoxy-arabonate is split by an aldolase into pyruvate and glycoaldehyde.^{7,8} Most of the genes and proteins involved in the fungal L-arabinose pathway were characterized in the two ascomycetes *Aspergillus niger* and *Trichoderma reesei*.⁴ In *T. reesei* L-arabinose reduction is mediated by the NADPH specific D-xylose reductase XYL1, which is the major reductase activity for the reduction of both pentoses D-xylose and L-arabinose.^{9,10} In *A. niger*, this NADPH-dependent reduction is accomplished by an L-arabinose specific LarA and a D-xylose specific XyrA.¹¹ The subsequent steps are mediated by L-arabitol 4-dehydrogenase,^{12,13} L-xylulose reductase,¹⁴ xylitol dehydrogenase,¹⁵ and xylulose kinase.¹⁶

Enzymes with L-xylulose reductase activity are found within the short chain dehydrogenase and reductase family¹⁷ and participate in the glucuronic acid/uronate cycle of mammals. In humans, LXR deficiency causes pentosuria, a clinically benign condition that results in large amounts of L-xylulose in the urine of such patients.¹⁸ The first fungal L-xylulose reductase, ALX1, was identified in the yeast *Ambrosiozyma monospora* and, interestingly, is NADH-dependent.¹⁹ Although an enzyme with L-xylulose reductase (LXR1) was described for *T. reesei*,²⁰ its functional characterization showed that it is actually a D-

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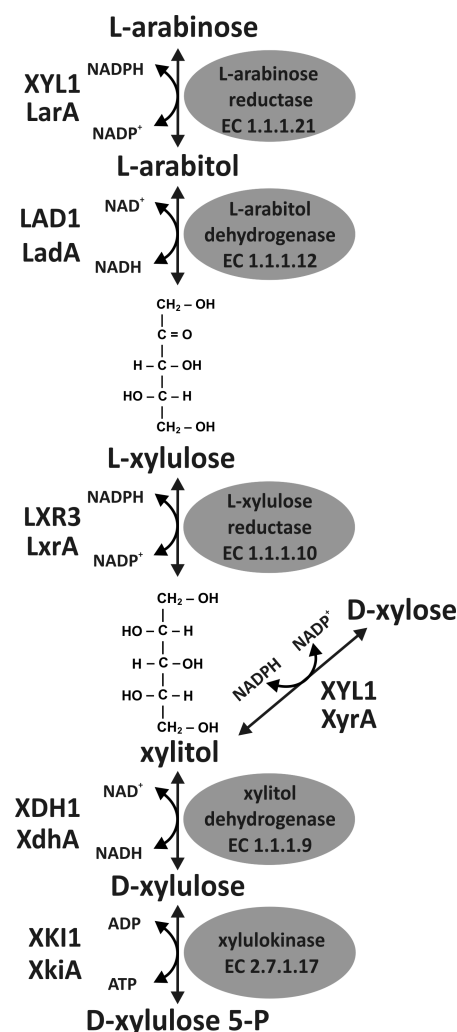


Figure 1. Fungal L-arabinose degrading pathway represented by enzymes of *A. niger* and *T. reesei*. The first three specific steps of the fungal L-arabinose catabolism lead to xylitol, the first common intermediate of the L-arabinose and D-xylose pathway. Xylitol is then converted to D-xylulose 5-phosphate before entering the pentose phosphate pathway. L-Arabinose reduction is mainly mediated by the D-xylose reductase XYL1 in *T. reesei*, while *A. niger* has a specific L-arabinose reductase LarA.

mannitol 2-dehydrogenase.^{21,22} Only recently was a true L-xylulose reductase LxrA identified in *A. niger*. Its deletion resulted in an almost complete loss of the NADPH specific L-xylulose reductase activity but had an only small effect on the growth on L-arabinose as the carbon source, explained by the presence of a NADH-dependent L-xylulose reductase activity.¹⁴ However, deletion of the LxrA homologue LXR4 in *T. reesei* showed that this gene is not involved in the oxidoreductive catabolism of L-arabinose but of D-galactose.²³

To clone putative LXRs involved in L-arabinose catabolism in *T. reesei*, we made use of the fact that all LXRs identified to date are found within the group of short chain dehydrogenases and reductases. Consequently, we screened the *T. reesei* genome database for SDRs encoding genes and reduced the number of LXR candidates by selecting for highly conserved fungal LXRs that are expressed in the presence of L-arabinose. Functional analysis identified a novel NADPH-dependent L-xylulose reductase that is involved in L-arabinose catabolism in *T. reesei*,

which is different from the case for the previously described enzymes.

MATERIALS AND METHODS

Strains and Growth Conditions. *T. reesei* QM9414 (ATCC 26921), $\Delta lxr2$, $\Delta tku70$,²⁴ and $\Delta lxr3$ were cultivated on malt extract agar supplemented with uridine (10 mM) when necessary. *Escherichia coli* JM109 (Promega) was used for plasmid construction. For liquid cultivations, 10⁶ spores per milliliter were incubated at 28 °C on a rotary shaker (250 rpm) in 250 mL of medium²⁵ in 1 L Erlenmeyer flasks containing 1% (w/v) of the indicated carbon source. For replacement cultivations, strains were pregrown for 24 h with glycerol as the carbon source, washed with sterile media without the carbon source, and transferred to new medium with the indicated carbon source. Mycelia for biomass measurements were washed and dried to a constant weight at 80 °C. Dry biomass data are the average of three separate biological experiments with a deviation of <15%. Growth on solid substrates was recorded by inoculating agar plates with a piece of pregrown agar in the center and measuring the colony diameter daily.

Screening for *T. reesei* Putative L-Xylulose Reductase-Encoding Genes. One hundred genes encoding SDRs are found in the *T. reesei* genome database (<http://genome.jgi-psf.org/Trire2/Trire2.home.html>). Their corresponding protein sequences were used in a BLASTP search against the NCBI database to identify highly conserved proteins in mycelial fungi (*e* value of <10⁻⁸⁰), followed by a BLASTP search to the genome database of the L-arabinose-utilizing yeast *Candida guilliermondii* (http://www.broadinstitute.org/annotation/genome/candida_guilliermondii; *e* value of <10⁻³⁰). The number of candidate LXRs was then further reduced by selecting those genes for which respective ESTs were found in the NCBI *T. reesei* EST database. The GenBank entries of the other four genes of the L-arabinose pathway are CB905315.1 (*xyl1*), CF883445.1 (*lad1*), CF944055.1 (*xdh1*), and CF878255.1 (*xki1*). LXR3 was deposited as GenBank entry BK008567.

Construction of Fungal Strains. For deletion of *lxr3*, ~1 kb of the *lxr3* up- and downstream regions were amplified with specific primers (Table 1). The downstream region was ligated into pGEM-T Easy (Promega) followed by the SpeI/XhoI restricted upstream region and the SalI restricted orotidine-5'-monophosphate decarboxylase-encoding gene *pyr4* as a selection marker,²⁶ resulting in pBM1. A 4.9 kb NotI *lxr3* deletion fragment was released from pBM1 and transformed into strain $\Delta tku70$ as described previously.²⁶ For reintroduction of *lxr3* into a $\Delta lxr3$ strain, the pyrithiamine resistance gene *ptrA* of *Aspergillus oryzae* was amplified from vector pME2892²⁷ with primers *ptrA_fw_PstI* and *ptrA_rv_HindIII* (Table 1) and ligated into pBluescript SK(+) (Stratagene). A 2.6 kb DNA fragment containing the whole *lxr3* coding region, ~1 kb of the upstream region, and 0.5 kb of the downstream region was amplified using the RELxr3-Acc65I/RELxr3-XhoI primer pair and introduced into the Acc65I and XhoI sites of this vector, resulting in pBM2. The Acc65I/HindIII fragment was used for transformation of $\Delta lxr3$ by electroporation.²⁸ The reintroduction of *lxr3* was verified by amplification of the 2.6 kb fragment by polymerase chain reaction (PCR) with oligonucleotides RELxr3-Acc65I and RELxr3-XhoI (data not shown). Deletion of *lxr2* (*tre54086*) was described previously.²⁴

Table 1. Oligonucleotides Used in This Study

oligonucleotide	sequence
ups- <i>lrx3</i> -Acc65I	<u>GGTACCGTCTTCAACTCCTGATAGGG</u>
ups- <i>lrx3</i> -XhoI	<u>CTCGAGGGTCGGAGATCAAGAAAG</u>
dws- <i>lrx3</i> -XhoI	<u>CTCGAGCAACAGAAAGAGGTAGACC</u>
dws- <i>lrx3</i> -XbaI	<u>TCTAGACAACCTTTAGCACCTGGAGC</u>
RElrx3-Acc65I	<u>GGTACCAACTCCTCGACCGAAATAG</u>
RElrx3-XhoI	<u>CTCGAGTCATGCTCATTGTGTGCTCC</u>
ptrA_fw_PstI	<u>TCTGCAGAAAGCTAGGAGATCGTCC</u>
ptrA_rv_HindIII	<u>TAAGCTTCTCTTGCATCTTTGTTTG</u>
rc- <i>lrx3</i> _HisN_fw_EcoRI	<u>ATATGAATTCACAATGCATCACCATC</u>
	ACCATCACGGGAAGAACGGCGCTTTCCG
rc- <i>lrx3</i> _rv_EcoRV	<u>TAATGATATCTCATGGCAGGCTGTAGCCGCC</u>
qPCR- <i>tef1</i> _fw	CCACATTGCCTGCAAGTTCGC
qPCR- <i>tef1</i> _rv	GTCGGTGAAGCCTCAACGCAC
qPCR- <i>xyl1</i> _fw	AGAACCTGGACAACACCTC
qPCR- <i>xyl1</i> _rv	GGCGGAGAAGTAGTTGTAG
qPCR- <i>lad1</i> _fw	GAGCGGTGTCATCGATCTATC
qPCR- <i>lad1</i> _rv	TCTTGGGATCTGCTGACGTCTC
qPCR- <i>lrx3</i> _fw	AACAGCTCCAAGGCCGCCGTGATTC
qPCR- <i>lrx3</i> _rv	AGACACGGTGTTGACGCGGGCAAAG
qPCR- <i>xdh1</i> _fw	GCATCTCGGCTGAGGACAAAC
qPCR- <i>xdh1</i> _rv	CGTGAATGCTCGTCTGGATC
qPCR- <i>lrx2</i> _fw	GCCGATATTGGAACAGACG
qPCR- <i>lrx2</i> _rv	GAAGACTGCGCCAATGTAC
qPCR- <i>tre122079</i> _fw	TCCAAGGCTGGTGTTCATGC
qPCR- <i>tre122079</i> _rv	ATCCAGCGGAGAGTGTGTTG

Nucleic Acid Isolation and Transcriptional Analysis.

Fungal mycelia were harvested by filtration, washed with cold tap water, frozen, and ground in liquid nitrogen. Following RNA isolation,²⁹ 5 μ g of total RNA was treated with DNase [DNase I, RNase free (Fermentas)] and reverse transcribed [RevertAid First Strand cDNA Kit (Fermentas)] using a 1:1 mixture of oligo-dT and random hexamer primers. To test for potential LXR-encoding genes, reverse transcription PCR (RT PCR) was performed with RNA isolated from *T. reesei* QM9414. Strain QM9414 was pregrown on medium containing glycerol as the carbon source followed by a transfer to new medium with L-arabinose, D-galactose, or D-glucose [1% (w/v)] as the carbon source. Data are found in Table S1 of the Supporting Information. Quantitative real-time PCRs (qPCRs) were performed by the iCycler iQ real-time detection system (Bio-Rad). Each reaction mixture contained 1 μ L of the 1:10 diluted cDNA (approximately 2.5 ng), 12.5 μ L of the iQ SYBR Green Supermix (Bio-Rad), primers (Table 1, final concentration of 100 nM), and nuclease free water in a final volume of 25 μ L. Primer efficiency was calculated using a dilution series from 1:1 to 1:1000 with the PCR baseline-subtracted mode. The threshold cycles (CT) were adjusted for an optimal efficiency of 2. The amplification protocol consisted of an initial denaturation step for 3 min at 95 °C followed by 40 cycles of denaturation (95 °C for 15 s), annealing, and elongation (61 °C for 20 s). qPCRs were conducted in triplicate. Data calculation was performed with iQ5 Optical System software version 2.0 (Bio-Rad) and REST.³⁰ Individual samples were normalized to the expression of *tef1* (translation elongation factor 1 α) as described previously.³¹

Phylogenetic Analysis. Phylogenetic analysis was performed using CLUSTALX version 1.8³² for protein sequence alignment, GENEDOC version 2.6³³ for visual adjustment, and MEGA version 5³⁴ for construction of phylogenetic trees.

Neighbor joining was used as the algorithm for distance calculation and evaluated by 1000 bootstrap rearrangements. To retrieve closely related SDR sequences from other species, *T. reesei* candidate SDRs were used in a BLASTP search against the NCBI database.

Recombinant Production and Purification of LXR3.

Expression, protein extraction, and purification of LXR3 in *S. cerevisiae* strain CEN.PK2-1D (European *S. cerevisiae* Archive for Functional Analysis) were performed as described for *A. niger* LxrA.¹⁴ An *lrx3* cDNA was amplified with primers rc-*lrx3*_HisN_fw_EcoRI and rc-*lrx3*_rv_EcoRV (Table 1) and cloned in pYX212 [URA3 selection (Ingenius R&D Systems, Madison, WI)], allowing expression of *lrx3* under the *TPI1* (triosephosphate isomerase) promoter to produce the recombinant LXR3 with an N-terminal His tag. The *lrx3* cDNA was verified by sequencing.

Preparation of *T. reesei* Cell Free Extracts. *T. reesei* mycelia grown in a liquid culture were washed and ground in liquid nitrogen. Per gram of mycelia (wet biomass) 3 mL of extraction buffer was added (PBS) [8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄ (pH 7.4), and 5 mM β -mercaptoethanol] and the mixture homogenized (12 \times 20 s, duty cycle of 25%, output of 2) with a Branson model 250 sonifier at 4 °C. After centrifugation (10000 rpm for 10 min at 4 °C), 20% glycerol (final concentration) was added and the cell free extracts were stored at -80 °C.

For the LXR activity measurements of *T. reesei* grown in the rich medium, 100 mL of YPG medium containing 10 g/L yeast extract, 2 g/L Bacto peptone, and 3% Difco gelatin (Becton Dickinson and Co.) was inoculated with 1 mL of the spore suspension. Overnight (16 h) growth at 28 °C resulted in a dense homogeneous mycelium suspension, which was collected by filtration and split into two comparable portions. The mycelia were resuspended in 50 mL of YP medium

supplemented with either 1% D-glucose or 1% L-arabinose and incubated for 6 h at 28 °C. For LXR activity measurements on minimal medium, 100 mL of medium containing 1% (w/v) glycerol was incubated for 24 h, and the mycelia were collected by filtration, split into two comparable portions, resuspended in 50 mL of MM medium supplemented with either 1% D-glucose or 1% L-arabinose, and incubated for 15 h at 28 °C. Following induction, mycelia were isolated by filtration and washed with water, and an appropriate amount of mycelia was transferred to a 2 mL tube with 0.6 mL of acid-washed glass beads (Sigma), 1 mL of lysis buffer [500 mM NaCl and 50 mM NaH₂PO₄ (pH 8.0)], and protease inhibitors (Complete, Roche). The cells were disrupted in a 30 s breaking session in a Precellys 24 instrument (Bertin Technologies). The cell extracts were clarified by centrifugation, and the supernatants were used in the enzyme assays. The protein concentration was measured using the Protein Assay kit (Bio-Rad).

Enzyme and Polyol Assays. The enzyme activity of cell free extracts was measured with a NanoPhotometer Pearl (Implen) or Helios Beta UV-vis spectrophotometer (Thermo Scientific) by recording the rate of change in absorbance at 340 nm for NAD(P)⁺ reduction and NAD(P)H oxidation. Polyol oxidation was performed in 100 mM Tris-HCl (pH 9.0) and 2 mM NAD(P)⁺ in the presence of 100 µg of cell free extracts and started with addition of 100 mM substrate. For sugar reduction, 100 mM HEPES-NaOH (pH 7.0) and 0.2 mM NAD(P)H were used.

Enzyme activity measurements of recombinantly produced proteins were performed by varying the substrate concentration over the range of 5–285 mM in 50 mM Tris-HCl buffer (pH 7.0) with 0.5 mM NADPH for sugar reduction and 100 mM Tris-HCl (pH 8.0) with 1 mM NADP⁺ for polyol oxidation. For analysis of the kinetic constants with NADPH, the activity was measured with varying NADPH concentrations over the range of 8–500 µM in 50 mM Tris-HCl buffer (pH 7.0) with 125 mM L-xylulose. Reactions were initiated by addition of the enzyme. The different substrate concentrations are indicated in the results. Enzyme assays were performed in microtiter plates (NUNC) with a Varioscan spectrophotometer (Thermo Electron Corp.). Activities are expressed in nanokatal and are given as specific activities (nanokatal per milligram of protein). High-performance liquid chromatography measurements were performed as described previously.^{10,21} Enzyme measurements for L-arabinose reductase, L-arabitol dehydrogenase, and xylitol dehydrogenase activity in cell free extracts were described previously.^{35,36}

RESULTS

Identification of Putative *T. reesei* L-Xylulose Reductases. All L-xylulose reductases characterized to date belong to the superfamily of short chain dehydrogenases and reductases (SDR). We therefore screened the *T. reesei* genome database for genes encoding putative LXRs and identified ~117 different SDRs. To reduce the number of putative candidate LXRs, we reduced their number by presuming the following: an L-xylulose reductase is a highly conserved enzyme, and therefore, orthologues should be present in the genomes of most mycelial fungi and present in the L-arabinose-utilizing yeast *C. guilliermondii*. Because the genes encoding the other four steps of the L-arabinose pathway in *T. reesei* are represented by ESTs in the NCBI database, we also tested if our potential LXRs are present in this EST database. To further reduce the number for functional analysis, we tested their expression by RT PCR

under L-arabinose inducing conditions and compared it to their expression on D-glucose. For seven genes, we found transcription under all conditions, but only three were specifically induced by L-arabinose. Because we originally also assumed that an LXR would be induced by D-galactose, we chose the two genes that showed on both sugars induction and termed them *lxr2* (*tre54086*) and *lxr3* (*tre60033*). An overview of the results of the in silico and expression analysis of 20 candidates is given in Table S1 of the Supporting Information.

Their transcriptional response to the presence of different inducers was then quantified by qPCR using *lad1* as a positive control for an L-arabinose inducible gene (Figure 2). *lxr3*

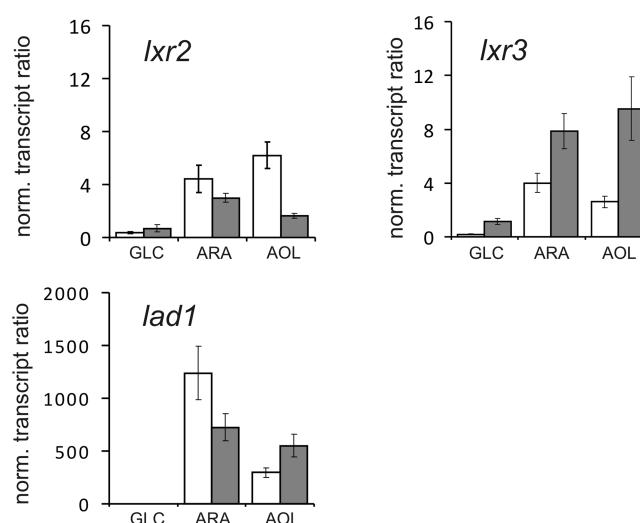


Figure 2. Transcriptional analysis of *T. reesei* *lxr2* and *lxr3*. *T. reesei* QM9414 was cultivated for 24 h on glycerol and replaced with new medium containing 1% (w/v) of the indicated carbon source (GLC, D-glucose; ARA, L-arabinose; AOL, L-arabitol) for 2 (gray bars) and 8 h (white bars). Expression of *lxr2*, *lxr3*, and *lad1* is related to their expression on glycerol after 24 h and normalized to the expression of *tef1*.

showed increased transcript levels when induced by L-arabinose and L-arabitol and an increase in transcript level from 2 to 8 h after replacement. *lxr2* also exhibited upregulation with highest transcript levels found at the earlier time point on L-arabinose or L-arabitol. In comparison to both *lxr2* and *lxr3*, *lad1* showed a higher inducibility on both L-arabinose and L-arabitol, which is due to its lower basal transcription level on glycerol.

Effect of Deletion of *lxr2* and *lxr3* on Growth. To test the potential role of *lxr3* in fungal L-arabinose catabolism, we produced a knockout cassette for *lxr3* in which the *lxr3* coding region was replaced by the *T. reesei* *pyr4* marker gene. Following transformation and analysis of the purified transformants by diagnostic PCR, several *lxr3* deletion strains were identified. The growth behavior of the Δ *lxr2*²⁴ and Δ *lxr3* strains was tested on different carbon sources. In this test, Δ *lxr2* strains showed no specific growth phenotype compared to its parental strain (Figure 3A). This was in contrast to Δ *lxr3* strains: here levels of growth on solid medium and biomass accumulation during liquid cultivation were strongly decreased for both L-arabinose and L-arabitol (Figure 3A,B). No effect, however, was found for growth with, e.g., D-glucose or D-xylose as the carbon source. A reintroduction of *lxr3* into the Δ *lxr3* strain restored growth on L-arabinose and L-arabitol (Figure 1 of the Supporting Information).

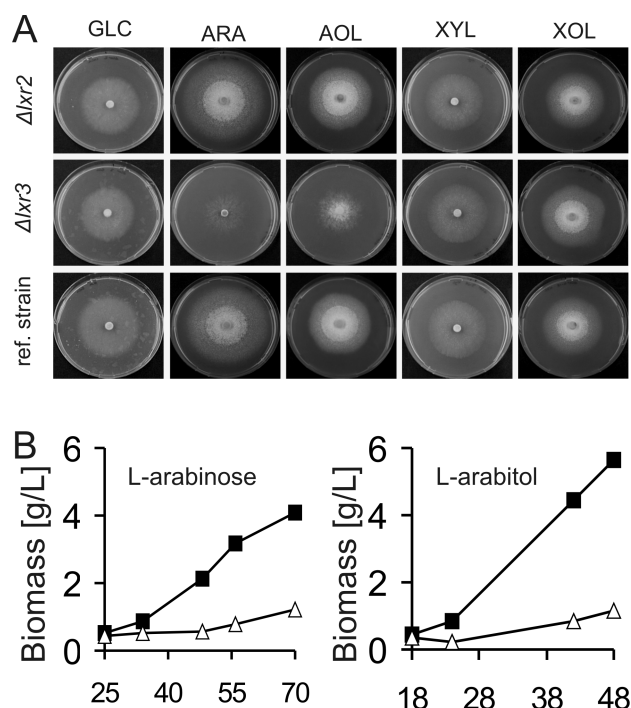


Figure 3. Effect of deletion of *lxl2* and *lxl3* on growth on different carbon sources. (A) Radial growth on agar plates after 3 days and (B) biomass accumulation during liquid cultivation on different carbon sources (1%, w/v) as indicated for *lxl3* (Δ) compared to the parental strain (\blacksquare): GLC, D-glucose; ARA, L-arabinose; AOL, L-arabitol; XYL, D-xylose; XOL, xylitol.

Deletion of *lxl3* Affects the Total L-Xylulose Activity and the Regulation of L-Arabinose Metabolism. The prominent effect of the *lxl3* deletion on the utilization of the carbon sources L-arabinose and L-arabitol was further investigated by determining the total L-xylulose reductase activity produced in cell free extracts in the $\Delta lxl3$ strain. L-Arabinose-induced cell free extracts were prepared from mycelia after replacement to minimal medium as well as rich medium with L-arabinose as the inducing carbon source. Deletion of *lxl3* led to a significant reduction in NADPH specific LXR activity after replacement to both media containing L-arabinose (Figure 4), while NADH specific LXR activity remained constant (e.g., 0.6 nkat/mg on L-arabinose-containing minimal medium). Again, the deletion of *lxl2* had no negative influence on LXR activity, indicating that LXR3 is responsible for the major NADPH specific L-xylulose reductase

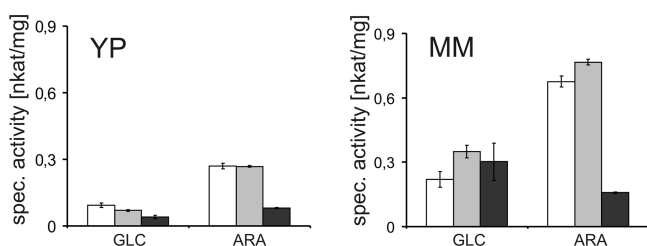


Figure 4. Effect of the deletion of *lxl2* and *lxl3* on total L-xylulose reductase activities. Mycelia were pregrown before the medium was replaced with rich (YP) or minimal medium (MM) containing either 1% (w/v) D-glucose or L-arabinose for 6 or 15 h. NADPH-dependent LXR activity was tested in crude protein extracts for QM9414 (white bars), $\Delta lxl2$ (gray bars), and $\Delta lxl3$ (dark gray bars).

in *T. reesei* during growth on L-arabinose as the carbon source. To examine if a deletion of *lxl3* has an influence on other genes involved in the L-arabinose catabolism, we performed further transcriptional studies. This analysis shows that the transcript levels of *xyl1* and *lad1* are considerably upregulated in the $\Delta lxl3$ strain during the whole cultivation period compared to that of the reference strain, while upregulation of *xdh1* is found only at a later time point around 48 h (Figure 5A). This change

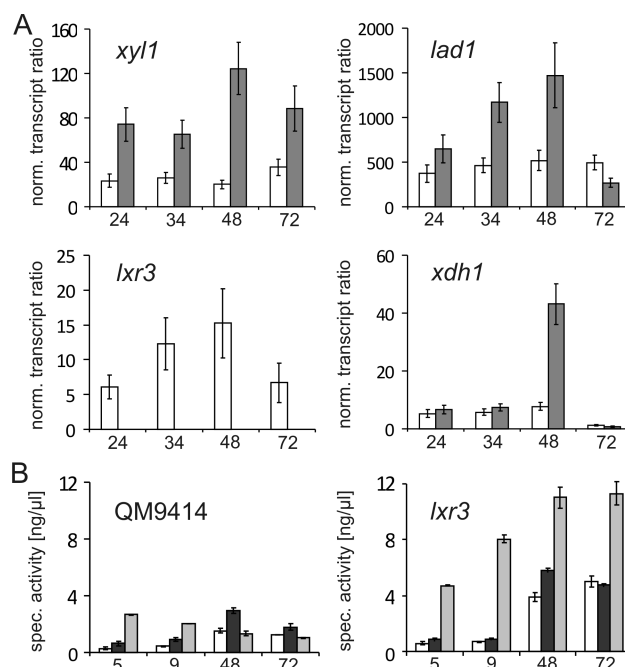


Figure 5. Consequences of the deletion of *lxl3* on the expression of other genes of the L-arabinose pathway. (A) Transcript levels of *xyl1*, *lad1*, *xdh1*, and *lxl3* relative to the expression during growth on glycerol at 24 h and normalized to *tef1*. (B) Total L-arabinose reductase (white bars), L-arabitol dehydrogenase (dark gray bars), and xylitol dehydrogenase (light gray bars) activities were measured in QM9414 and $\Delta lxl3$. Strains were either precultivated on glycerol and replaced with L-arabinose (5 and 9 h) or cultivated directly on L-arabinose (48 and 72 h).

in the transcription profile was also reflected by the elevated total enzyme activities for L-arabinose reductase, L-arabitol dehydrogenase, and xylitol dehydrogenase in the cell free extracts (Figure 5B).

Characterization of *T. reesei* LXR3. Functional analysis supports the role of LXR3 in L-arabinose catabolism. To characterize the enzyme with respect to its L-xylulose reductase activity, we expressed LXR3 recombinantly in *S. cerevisiae* and investigated substrate specificities and enzyme kinetics. The purified enzyme reduced L-xylulose with a K_m of 16 mM, a V_{max} of 367 nkat/mg, and a k_{cat} of 11.4 s⁻¹. For NADPH, we obtained a K_m of 0.13 mM, a V_{max} of 250 nkat/mg, and a k_{cat} of 7.75 s⁻¹.

LXR3 also exhibited activity with D-ribulose (K_m = 105 mM; V_{max} = 266 nkat/mg; k_{cat} = 8.24 s⁻¹) and with polyols D-sorbitol (K_m = 250 mM; V_{max} = 58 nkat/mg; k_{cat} = 1.8 s⁻¹) and xylitol (K_m = 100 mM; V_{max} = 33 nkat/mg; k_{cat} = 1 s⁻¹) and weak activity with D-xylulose, L-sorbose, and D-fructose (V_{max} < 30 nkat/mg). No activity was recorded with L-xylo-3-hexulose, the substrate of LXR4 in the oxidoreductive D-galactose pathway,²³ D-sorbose, D-ribitol, D-arabitol, or L-arabitol. The enzyme was

also strictly NADP(H) specific, and no activity was observed with NADH as the cosubstrate.

Phylogenetic Analysis of L-Xylulose Reductases. The fact that *T. reesei* LXR3 is quite dissimilar from *A. niger* LxrA, while both are in vivo functional L-xylulose reductases, prompted us to investigate their phylogenetic relationship. We also included the other three *T. reesei* LXR proteins, LXR1 (D-mannitol 2-dehydrogenase, which also exhibits L-xylulose reductase activity), LXR2, and LXR4 (L-xylulose-3-hexulose reductase), and used them as a query in a BLASTP search against the NCBI database. The resulting best hits were pruned from duplicates, and 182 protein sequences were subjected to a neighbor joining analysis and rooted to the corresponding ALX1 from *Am. monospora* and two other proteins from different yeasts. The result (Figure 6 and Figure 2 of the

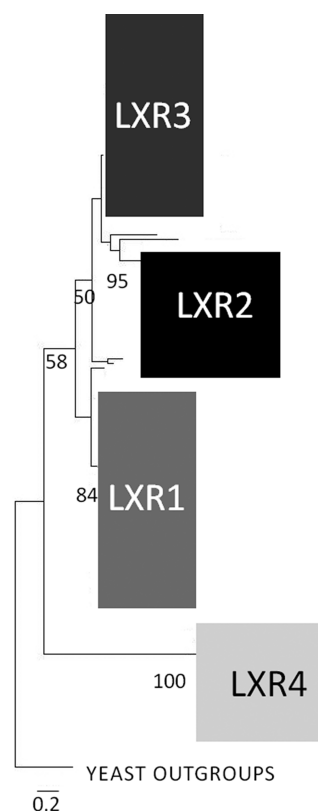


Figure 6. Scheme of the phylogenetic relationship of *T. reesei* LXR3 to other in vivo functional L-xylulose reductases, including the L-xylulose reductase LxrA of *A. niger* and ALX1 of *Am. monospora* found in yeast outgroups. Also included are LXR1 (D-mannitol 2-dehydrogenase, which also exhibits L-xylulose reductase activity), LXR2, and the L-xylulose-3-hexulose reductase LXR4. The numbers below nodes indicate the bootstrap value. The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions. The detailed phylogenetic tree is found in Figure 2 of the Supporting Information.

Supporting Information) shows that the fungal LXR proteins form three major clades: one basal clade leading to a large clade that contained the *A. niger* functional L-xylulose reductase LxrA and the L-xylulose-3-hexulose reductase LXR4, another that contained LXR1, and a third that was split into two subclades containing LXR2 and LXR3. Branches within the LXR3 clade showed poor bootstrap support and displayed paralogs in many species, particularly Pyrenomycetes (also *Trichoderma virens* and *Trichoderma atroviride* but not *T. reesei*). This suggests that

L-xylulose reductases and related enzymes have proliferated in Pyrenomycetes and thereby apparently adapted their substrate specificity. From this analysis, it is obvious that the trait for L-xylulose reductase has evolved independently within the family of short chain dehydrogenases for enzymes of the L-arabinose pathway and the glucuronic acid pathway and that even the fungal LXRs involved in L-xylulose reduction in the L-arabinose catabolic pathway have evolved in different clades of SDRs.

DISCUSSION

Most of the genes and their corresponding enzymes involved in the L-arabinose and D-xylulose pathway have been characterized from the two ascomycetes *A. niger* and *T. reesei*. Although the overall sequence of reactions consisting of four oxidoreductive steps and a final phosphorylation is conserved (Figure 1), a comparative functional analysis reveals a number of species specific adaptations such as the presence of a single enzyme (XYL1) for both L-arabinose and D-xylulose reduction in *T. reesei*, but two rather specific reductases for L-arabinose (LarA) and D-xylulose (XyrA) in *A. niger*.^{11,36} Here we identify a further difference and show that *T. reesei* uses a novel L-xylulose reductase LXR3 in L-arabinose catabolism.

In the past, different enzymes responsible for L-xylulose reduction were identified in the family of SDRs. The first enzymes responsible for L-xylulose reduction were found in mammals, where their absence blocked the pathway for D-glucuronic acid leading to the accumulation of L-xylulose in blood and urine. The molecular background of this condition, pentosuria, was recently elucidated.¹⁸ To date, two fungal LXRs have been functionally verified to be involved in L-arabinose catabolism, including the NADH-dependent ALX1 of the yeast *Am. monospora*¹⁹ and the recently identified NADPH-dependent LxrA of *A. niger*.¹⁴ Although it was assumed for many years that the LXR step is NADPH-dependent,^{3,37} recent investigations in *A. niger* showed that the NADPH-dependent L-xylulose reductase activity is not needed for rapid growth on L-arabinose. The situation is obviously different in *T. reesei* where the strongly reduced NADPH-dependent L-xylulose activity as a consequence of *lrx3* deletion leads to a severe reduction in the level of growth on L-arabinose and L-arabitol as carbon sources. The question of whether the NADH-dependent activity in *T. reesei* is too low to replace the NADPH-dependent activity as suggested for *A. niger* remains.^{14,37} Future research will clarify if a NADH-dependent LXR is responsible for the major conversion of L-xylulose to xylitol in *A. niger*. A NADH-dependent L-xylulose reductase step would also have consequences for the redox balance. Although the overall process of L-arabinose assimilation is redox neutral, it leads to an unequal use of cofactors with two reductive NADPH-dependent and two oxidative NAD⁺-dependent steps. Interestingly, the V_{\max} of purified LXR3 is much lower than that of purified *A. niger* LxrA ($V_{\max} = 10833.3 \text{ nkat/mg}$)¹⁴ but higher than that of purified LXR1 (75 nkat/mg).²⁰

The L-xylulose reductases and related proteins appear to have undergone an intriguing evolution: the presence of members of Eurotiomycetes, Dothidiomycetes, and Pyrenomycetes in both large phylogenetic clades suggests that there have been early duplication events that were followed by gene losses in the former two classes with the exception of the Pyrenomycetes where the gene losses were less intense. In addition, the substrate specificity of these proteins appears to have undergone readjustment as, e.g., LXR3 is not able to convert L-xylulose-3-hexulose whereas LXR4 is able to do so.²³ As a

consequence of this evolution, *T. reesei* L-xylulose reductase LXR3 is more closely related to *T. reesei* D-mannitol dehydrogenase LXR1 than to *A. niger* L-xylulose reductase LxrA. Originally, we assumed that LXR3 might also be responsible for the conversion of L-xylulose-3-hexulose, the product of LAD1,¹³ to the corresponding polyol D-sorbitol in the oxidoreductive D-galactose pathway. This would be analogous to the findings that in *T. reesei* other L-arabinose pathway enzymes such as XYL1 and LAD1 function in this oxidoreductive D-galactose catabolism. However, our results show that LXR3 is not able to convert L-xylulose-3-hexulose. We have recently identified yet another SDR LXR4 that is involved in this step in oxidoreductive D-galactose catabolism.²³

A major consequence of the *lcr3* deletion is the disturbance of L-arabinose catabolism. Its deletion results in a specific upregulation of genes of the L-arabinose pathway acting upstream of *lcr3*, i.e., *xy11* and *lad1*, while *xdh1* that is responsible for the step downstream of *lcr3* is only upregulated to a later time point. This would imply that the inducer for L-arabinose catabolic genes *xy11* and *lad1* is produced upstream of *lcr3*, while the inducer for the upregulation of *xdh1* accumulates at a later time point.

■ ASSOCIATED CONTENT

■ Supporting Information

Overview of the *in silico* and expression analysis of the 20 candidates (Table S1), the complementation of *lcr3* in a $\Delta lcr3$ strain (Figure 1), and a detailed phylogenetic tree of different LXR proteins (Figure 2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS

SDRs, short chain dehydrogenases and reductases; LXR, L-xylulose reductase; ESTs, expressed sequence tags.

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