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DISSERTATION

The regulation of hemicellulose metabolism and its metabolite pools in filamentous fungi

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Content

Abstract

Hemicelluloses are complex heterogenous polysaccharides that beside cellulose and pectin contribute to the main parts of plant cell walls. Among the microorganisms capable of hemicellulose degradation the saprobic ascomycete *Trichoderma reesei* represents a paradigm for the biochemistry and regulation of fungal hemicellulose degradation. Moreover, the extracellular secreted xylanases find applications in different industrial processes including biobleaching of kraft pulp, as additive to dough and animal feed and filtration of juices or in the depolymerization of plant cell walls for biofuels and different biorefinery products. Optimizing xylanase production in industrial fungal strains requires exact knowledge about the basic molecular mechanisms that underlie regulation and expression of the xylanase genes. This work adresses numerous questions of hemicellulose metabolism and regulation, *i.e.* a survey on *T. reesei* xylanases, the intracellular degradation of hemicellulose derived sugars resulting from enzymatic decomposition by different hemicellulases and the inducer formation for these enzymes within the catabolic pathways. The *T. reesei* genome harbors at least five xylanases including the GH11 family xylanases *xyn1, xyn2* and *xyn5,* GH10 family *xyn3* and GH30 xylanase *xyn4*. The major hemicellulosic pentoses D-xylose and L-arabinose function as independent inducers that can act in a synergistic manner to trigger the expression of some of the xylanase genes. Transcriptional regulation is further influenced by the carbon catabolite repressor CRE1 and the general activator XYR1. Interruption of the first two catabolic steps in the degradation of D-xylose and L-arabinose enhances induction levels of the main xylanases. With respect to the intracellular catabolism two missing links in the degradation of L-arabinose and D-galactose were identified. These include a novel short-chain reductase with a key role in the oxido-reductive D-galactose degradation pathway which is in *T. reesei* encoded by the L-xylo-3-hexulose reductase *lxr4* and in *Aspergillus niger* by *xhrA*. Both enzymes catalyze the conversion of L-xylo-3-hexulose to D-sorbitol using NADPH as cofactor but are phylogenetically not closely related. Deletion of these genes resulted in the inability of both fungi to grow on galactitol as sole carbon source. In L-arabinose catabolism the *T. reesei* LXR3 was identified as a novel enzyme responsible for NADPH dependent L-xylulose to xylitol reduction. This enzyme is again not closely related to its *A. niger* counterpart LxrA. These new insights on the regulation of xylanases and on the biochemistry of the different

monosaccharide catabolic pathways significantly contribute to our understanding of the complexity of hemicellulose degradation, metabolism and enzyme regulation in filamentous fungi and show that fungi have evolved different strategies to regulate the extracellular hemicellulases and intracellular assimilation of hemicellulose derived monosaccharides.

Kurzfassung

Hemizellulose ist ein Sammelbegriff für komplexe heterogene Mehrfachzucker, welche neben Zellulose und Pektin den größten Teil pflanzlicher Zellwände ausmachen. Einige spezialisierte Mikroorganismen sind im Stande effizient Hemizellulose abzubauen und zu verwerten. Darunter befindet sich der saprobe Ascomycet *Trichoderma reesei*, welcher als ein Modellsystem für Biochemie und Regulation des Hemizelluloseabbaus in Pilzen gilt. Die extrazellulär sekretierten Xylanasen und Zellulasen dieses Pilzes finden in einem breiten Spektrum an industriellen Prozessen Anwendung, unter anderem im Biobleichen von Zellstoff, als Zusatz in Teig- und Tierfuttermitteln und in der Filtration von Fruchtsäften, sowie im Abbau von Pflanzenbiomasse zu einfachen Zuckern, die weiter zu Biokraftstoffen oder verschiedenen Bioraffinerieprodukten umgesetzt werden können. Die gezielte Optimierung der Xylanaseproduktion industrieller Pilzstämme erfordert ein umfassendes Wissen über die molekularen Mechanismen, die der Regulation und Expression der Xylanasegene zu Grunde liegen. Die vorliegende Arbeit behandelt verschiedene Fragestellungen betreffend Hemizellulosemetabolismus und –regulation. Die Xylanasen in *T. reesei* und deren Induzierbarkeit durch Metabolite, die im Zuge der intrazellulären Verstoffwechselung von Abbauprodukten langkettiger Hemizellulosen entstehen, werden dabei umfassend beleuchtet. Das Genom von *T. reesei* umfasst mindestens fünf Xylanasen. Dazu zählen die der GH11 Familie zugehörigen *xyn1, xyn2* und *xyn5*, die GH10 Xylanase *xyn3* und *xyn4*, welche der GH30 Familie zugeordnet wird. D-xylose und L-arabinose, die am häufigsten in Hemizellulose vorkommenden Pentosen, induzieren unabhängig voneinander die Expression dieser Xylanasegene und zeigen einen synergistischen Effekt betreffend der Induktion einiger Xylanasen. Die transkriptionelle Regulation der Xylanasegene wird des Weiteren über den Aktivator XYR1, sowie über Kohlenstoff Katabolit Repression durch den Transkriptionsfaktor CRE1 erreicht. Eine Unterbrechung der ersten beiden enzymatischen Schritte im Stoffwechselabbauweg von D-Xylose und L-Arabinose verstärkt die Expression der für den Abbau von Arabinoxylan wichtigsten Xylanasen. In Bezug auf den intrazellulären Katabolismus konnten im Zuge dieser Arbeit zwei bis dato unbekannte Enzyme identifiziert und charakterisiert werden, die eine maßgebliche Rolle im Abbau von L-Arabinose und D-Galactose spielen. Dies ist zum ersten eine kurzkettige Dehydrogenase, welche eine Schlüsselrolle im oxido-reduktiven D-Galactose Abbau spielt und in *T. reesei* durch das L-Xylo-

3-hexulose 4 Gen *lxr4* und in *A. niger* durch *xhrA* kodiert wird. Beide Enzyme katalysieren die Reaktion von L-Xylo-3-hexulose zu D-Sorbitol mittels NADPH als Cofaktor, jedoch sind sie nicht nahe miteinander verwandt. Die Deletion dieser Gene resultiert im Verlust der Fähigkeit auf Galactitol als einziger Kohlenstoffquelle zu wachsen. Im L-Arabinose Stoffwechselweg von *T. reesei* wurde LXR3 als neues Enzym identifiziert, welches abhängig von NADPH L-Xylulose zu Xylitol reduziert. Auch diese kurzkettige Reduktase ist nicht nahe mit seinem Gegenstück LxrA in *A. niger* verwandt. Zusammengefasst tragen diese neuen Einblicke in die Mechanismen der Xylanaseregulation und die Biochemie der Stoffwechselwege verschiedener hemizellulosestämmiger Monosaccharide signifikant dazu bei, die Komplexität des enzymatischen Hemizelluloseabbaus durch filamentöse Pilze besser zu verstehen und zeigen, dass Pilze im Laufe der Evolution unterschiedliche Strategien entwickelt haben, ihre extrazellulären Hemizellulasen zu regulieren und die Abbauprodukte der Hemizellulosen zu verstoffwechseln.

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General Introduction

Hemicelluloses beside cellulose are the second most abundant biopolymers on earth. These complex polysaccharides are the main compounds of plant cell walls. Together with pectins and in wooden species also lignin they help to strengthen and stabilize the cell and offer protection from parasitic decay. A number of microorganisms have developed different strategies to break down these recalcitrant biopolymers and to convert them into smaller compounds ready for assimilation. These organisms posses a broad arsenal of highly specialized enzymes that are secreted and act usually synergistically in order to degrade long-chain cellulose and hemicelluloses into their basic building units, including different mono- and disaccharide sugars. Saprobic fungi form the biggest group of organisms that are capable to decompose plant biomass. Massive amounts of carbon originated from dead plant material are degraded by fungi every year, making these microorganisms fundamental players in the world's carbon cycle.

As carbohydrolytic enzymes find applications in a broad range of industrial processes research focused on understanding the complex molecular mechanisms that underlie the expression of these enzymes in selected model microorganisms with the target to generate high enzyme producer strains by random mutagensis and genetic engineering, respectively. Carbohydrate-active enzymes (CAZymes) that degrade degrade, modify, or create glycosidic bonds have been classified based on sequence similarities into different families including the glycoside hydrolase families (GHs). GH families and members are listed in the CAZymes database (www.cazy.org) that has been developed by Bernard Henrissat and coworkers in Marseille (Cantarel *et al.* 2009).

Fungi

Beside plants and animals, fungi form a large kingdom within the domain of the eukaryots. They represent a huge diversity in physiology and morphology, as well as ecology. Some species adapted to extreme living conditions like high temperatures, high pressure, aridity or acidic habitats (Seckbach 1999). To date over 80.000 fungal species are known but the expected number of existing species lies at around 1 - 1.5 million (Hawksworth 2001; Mueller and Schmit 2007). A distinction is made between *Eumycota*, also referred to as 'real

fungi', *Myxomycota* and *Oomycota*. The *Eumycota* are divided into the subgroups *Chytridiomycota, Glomeromycota* and the largest groups *Ascomycota, Zygomycota* and *Basidiomycota* and are characterized by the possession of a complex chitin containing cell wall (Nash 1996). Cells can either exhibit unicellular growth (*i.e.* yeasts) or multicellular forming hyphae. These long, branching filaments form the mycelium (*i.e.* filamentous fungi). Fungi influence the human life in many ways. Pathogenic fungi harm human health by production of mycotoxins (*e.g.* aflatoxin by *Aspergillus flavus*) or direct cultivation of skin, mucosa (*e.g. Trichophyton rubrum, Candida albicans*) or as final stage systemical infection (mycosis) of immunocompromised people (Kozinn and Taschdjian 1966; Novick *et al.* 1987). In addition, especially crop pathogenic fungi can be responsible for massive loss of yield and decay of food stocks (*e.g. Botrytis cinerea, Fusarium ssp.*) (Punja and Utkhede 2003). On the other hand fungi play an important role in numerous biotechnological processes since ancient times. Yeasts such as *Saccharomyces cerevisiae* found applications in brewing and baking. Within the last years improved producer strains which were generated by random mutagenesis and genetic engineering, respectively enable an efficient industrial production of antibiotics, vitamins, citric acid and other valuable (bio)chemicals. An advantage of many fungi over e.g. bacteria is that they produce large amounts of extracellular enzymes. Especially, the high scale production of different plant cell wall degrading enzymes including pectinolytic, xylanolytic and cellulolytic enzymes has received considerable interest. These enzymes find application in numerous industrial processes including kraftbleaching and cotton bleaching by removing chromophores from the fibers, improvement of dough quality and taste, clarification of must and juices and additives in animal feed. Current attemps to convert biomass to biofuels also involve beside cellulases also the use of xylanases (Biely 1985; Viikari *et al.* 1994; Beg *et al.* 2001; Csiszár *et al.* 2006; Butt *et al.* 2008).

Trichoderma reesei

The genus *Trichoderma* has been introduced by Persoon in 1794 (Persoon 1794). *Trichoderma spp.* belong to the subphylum *Pezizomycotina*, class *Sordariomycetes*. Until 2006 over 100 species have been characterized within the genus *Trichoderma/Hypocrea*. Species of the genus *Trichoderma* are economically important organisms. *T. reesei* is mainly used for enzyme production but *T. harzianum, T. atroviride* and *T. virens* for example are

used as mycoparasitic biocontrol agents and bio fertilizers as they were shown to promote plant growth (Harman and Kubicek 1998).

The first isolate QM6a (Quarter Master 6a) of the species *T. reesei* has been taken from cotton canvas of US army tents on the Solomon Islands during World War II in an attempt to identify the organism and the mechanism responsible for textile degradation in this area (Reese 1976). A link between *T. reesei* and the sexually reproducing species *Hypocrea jecorina* has been proposed in 1865 (Tulasne and Tulasne 1865) and could be confirmed by *in silico* analysis about a hundred years later (Kuhls *et al.* 1996). *T. reesei* had been considered as strictly asexually reproducing until in 2009 Seidl and co-workers described *T. reesei* to be heterothallic and succeeded to cross QM6a (*MAT1-1*) with an *H. jecorina* wild-type isolate of the opposite mating type (*MAT1-2*). However, they also found out that *T. reesei* QM6a is female sterile which still prevents sexual crossing between QM6a and QM6a strains with an inverted mating type (Seidl *et al.* 2009).

The fungus and especially its mutants have become of increased commercial interest because of their ability to produce high amounts of extracellular cellulolytic enzymes. It has been classified as safe organism since it does not produce mycotoxins or any other compounds pathogenic to humans, a fact that makes it also an appropriate host for the production of heterologous gene products like calf chymosin or IgG1 antibody (Harkki *et al.* 1989; Nyyssönen *et al.* 1993; Nevalainen *et al.* 1994). Based on the original single isolate QM6a a broad variety of mutants has been created for improved extracellular enzyme yield. By using linear electron acceleration the cellulolytic overproducer strains QM9123 and in succession QM9414 were generated that are able to produce twice as much cellulases as QM6a (Mandels *et al.* 1971). However, carbon catabolite repression is often responsible for drastic loss of enzyme yield in industrial fermentation processes caused by the presence of repressing compounds (*e.g.* glucose) in plant biomass. Hence new screening approaches for the identification of strains exhibiting decreased carbon catabolite repression were adopted. Targeted selection of cultivars that exhibit both high carbohydrolytic enzyme production and decreased carbon catabolite repression after three rounds of random mutagenesis by UV and N-nitroguanidine treatments led to the identification of the *T. reesei* strain RUT-C30 (Montenecourt and Eveleigh 1977a; Montenecourt and Eveleigh 1977b; Montenecourt and Eveleigh 1979). This strain is often used as a starting point for many industrial enzyme producers.

The genome of *T. reesei* comprises seven chromosomes carrying 9129 genes. The genome has a total size of 33.9 Mb and 40.4% of them refer to coding regions (Martinez *et al.* 2008). Interestingly, with 200 the total number of glycoside hydrolase genes in *T. reesei* is remarkably lower than in other filamentous fungi e.g. *Aspergillus nidulans* (247 GH genes) or *Aspergillus oryzae* (285 GH genes) (Martinez *et al.* 2008). However, while the number of genes encoding cellulases (10) in the secretome resembles to that of other Pezizomycotina, *T. reesei* has only a limited number of pectinases and lacks pectin lyases or pectin esterases. *T. reesei* possesses also a lower number of polysaccharide monoxigenases in comparison to other fungi. Anyway *T. reesei* features a broad arsenal of hemicellulolytic enzymes such as α-arabinofuranosidases and α-galactosidases (Druzhinina *et al.* 2012).

Aspergillus niger

Aspergillus niger comprises a group of soilborne ascomycetes that belong to the class of sordariomycetes and live on dead plant material. The genus *Aspergillus* was mentioned for the first time in 1729 and is characterized by its aspersorium shaped conidiophor that in the case of *A. niger* is black coloured (Micheli 1729). There is a large phenotypic and genotypic versatility between different *A. niger* strains. *A. niger* was first described by van Tieghem in 1867 as producer of the tannic acid cleaving enzyme tannase (van Tieghem 1867). From the beginning of the 19th century it started being used for the production of organic acids such as citric, gluconic and fumaric acid. The first strain patented for citric acid fermentation, ATCC 1015, has been considered as wild type strain (Baker 2006).

Over the last decades *A. niger* also became one of the most widely used industrial producers of enzymes such as glucoamylase, protease and feruloyl esterase and heterologous expressed proteins (Withers *et al.* 1998; Record *et al.* 2003; Lubertozzi and Keasling 2009). For these purposes *A. niger* strain CBS 513.88, that originates from the glucoamylase A overproduction strain NRRL 3122 is used (van Lanen and Smith 1968). Comparison of the genome sequences of both strains revealed chromosomal rearrangements and inversions, mutations in amino acid synthesis pathway, metabolism and electron transport chain genes. The genome size of ATCC 1015 is 34.85 Mb with 11200 genes. CBS 513.88 comprises a genome of 33.93 Mb and 14165 genes (Pel *et al.* 2007; Andersen *et al.* 2011). *A. niger* has

been considered safe by the United States Food and Drug Organisation as only a low percentage of strains produce the harmful ochratoxin A (Schuster *et al.* 2002).

Industrial applications of cellulases and xylanases

The carbohydrolytic enzymes secreted by fungi are used for different purposes in various types of industries. On overall cellulases, hemicellulases and pectinases together contribute to 20% of the World's enzyme market (Polizeli *et al.* 2005). The most common application for xylanases in pulp and paper industry is the biobleaching of softwood derived kraft pulp. 90% of the lignin that is responsible for the brown color of the wood is destroyed in an initial alkaline treatment under high temperature. The residual hemicelluloses are degraded by enzyme treatment, ideally using a mixture of β-1,4-xylanases and β-1,4-mannanases. In addition the enzymes cleave the linkages between lignin and hemicellulose molecules leading to release of lignin and thus brightening of the pulp. Hence the application of expensive and toxic chlorine based bleaching reagents can be reduced up to 25% (Bajpai and Bajpai 1992; Viikari *et al.* 1994). Other sectors that profit from the use of hemicellulosic enzymes are food and animal feed industries. Wheat and rye flour bread-making industries include β-1,4-xylanases to enhance bread quality and taste (Courtin and Delcour 2002; Shah *et al.* 2006). Further, the digestibility of especially cereal based animal feed can be improved by xylanase supplemental. In particular poultry performance was shown to improve significantly using an enzyme accompagnied diet because the nutritional value of feed increases by higher accessability of proteins and fat (Marquardt *et al.* 1994).

Another important issue that in the near future will gain more and more relevance is the development of renewable energy and carbon sources. Beside biodiesel bioethanolappears as promising solution as it produces less carbon monoxide than gasoline upon combustion.The International Energy Agency (IEA) demands to reduce CO_2 emissions to 50% by 2050. To achieve this goal the use of biofuels should increase dramatically from an actually provided 2% to 27% of the World's total transport fuel (OECD/IEA 2011). Bioethanol that is produced from sugar cane, corn, wheat and related feedstocks is referred to as first generation bioethanol. Direct competition with food crop cultivation and the need for expansion of agricultural areas for feedstock growth led to ever stronger criticism from society. As a result a more sustainable second generation system of bioethanol production

was introduced that involves mainly agricultural plant waste materials, straw and energy crops such as *Miscanthus* and switchgrass as feedstocks. A critical obstacle in the ethanol production process is the lignin that hinders proper enzymatic decomposition of cellulose and hemicelluloses.

Thus contemporary applied energy research focuses on the development and improvement of the biomass pretreatment process. There are different pretreatment processes available that cause decomposition of the rigid cell wall structures and are commonly combined with enzymatic treatments: mechanical treatment (hammer mill, ball mill), temperature treatment (steam explosion, low temperature steep delignification) or chemical treatment (alkaline treatment) (Zheng *et al.* 2009). Pretreatment methods and composition of the enzyme mixture are adapted depending on the type of raw material. Xylanases find application in this process as part of complex enzyme premixes. Fermentation is mainly carried out by established yeast strains that are continuously adapted to this process *e.g.* via metabolic engineering. In Austria there is one bioethanol production plant run by Agrana Bioethanol GmbH.

Beside energy, diverse value added chemicals can be produced from plant biomass as raw material in biorefineries that work analogous to petrol refineries. To date five commercial and twelve pilot plants have been e.g. established all across the United States of America in the course of the US biomass program (U.S. Department of Energy, *http://energy.gov*).

Composition of the plant cell wall

All terrestrial plants possess cell walls, dynamic complexes of carbohydrates and proteins with numerous functions including contribution to strength and stability of the cell, physical protection against pathogens, water transport and control of cell growth rate and direction. In addition it has been shown that compounds of the cell wall are involved in signalling and metabolic pathways. Depending on the plant species and the developmental stage of the plant, structure and composition of the cell wall varies. The primary cell wall that is present in growing and dividing cells is distinguished from the secondary plant cell that encloses mature plant cells. Hemicelluloses make up to 30% of the primary walls dry weight while cellulose plays a minor role. In contrast secondary walls are characterised by a high level of cellulose and hemicelluloses are present to a lower level. Over hundred of β-1,4-linked

glucose units form a cellulose chain and about 30-40 of these chains are bundled to a microfibril. The crystalline unsoluble microfibrils are embedded in a matrix of pectin, glycoproteins and in case of especially wooden plants also lignin. The exact mechanism of how cellulose microfibrils are interconnected is not known but there are a few models available that propose hemicelluloses, either alone or in combination with other matrix polymers, as linkers (Cosgrove 2005).

Tab. 1. Composition of plant cell walls of different plant sources (Betts *et al.* 1991).

Source	Cellulose	Hemicellulose	Lignin
Hardwood	45-55%	24-40%	18-25%
Softwood	45-50%	25-35%	25-35%
Grasses	25-40%	25-50%	10-30%

Hemicelluloses

Hemicellulose is the overall term for a large group of heterogenous polysaccharides that depending on their backbone structure, number and type of substitutions are divided into different subgroups that appear in a huge variety of compositions and complexities. Through their numerous reactive branches they easily establish stable connections to other polymers of the plant cell wall matrix thus contributing to stability and protection of the plant cell. In addition hemicelluloses function as storage substances in seeds and prevent them from drying (Dey 1978; Bewley and Black 1994; Buckeridge *et al.* 2000). On average hemicelluloses make up 20% of a plant's dry weight. The most common type of these complex polymers in nature is xylan characterized by a backbone that is composed of β-1,4-linked D-xylose units. In rare cases such as marine algae species (e.g. *Caulerpa filiformis*) also β-1,3 linkages occur (McCandless 1981). Linear xylan molecules have been isolated from the grass esparto (Chanda *et al.* 1950) but in general decorations of mono- and disaccharidic sugars are prevalent. Single or multiple L-arabinofuranosyl units appear α-1,2 or α-1,3 connected to the backbone in the case of arabinoxylan. In addition α-D-glucuronic acid residues may be connected over an α-1,2 linkage to D-xylose (glucuronoxylan or glucuronoarabinoxylan). Xylans are the major hemicelluloses in cereals and hardwood while

the most common hemicelluloses in gymnosperm cell walls (12-15%) are mannan based polymers such as galactomannan, glucomannan or galactoglucomannan. In contrast to xylan the backbone of these molecules is built up by β-1,4-linked D-mannose or randomly distributed D-mannose and D-glucose units which may carry branches of α-1,6 linked D-galactose. However the major component of primary plant cell walls is xyloglucan (around 20%). The backbone of xyloglucan is formed by β-1,4-linked D-glucopyranose units and based on their substitution pattern the two types XXXG and XXGG are distincted. In type XXXG three D-glucose units in a row carry α-1,6 linked D-xylopyranosides that often are further α-1,2 linked to D-galactose and L-fucose.

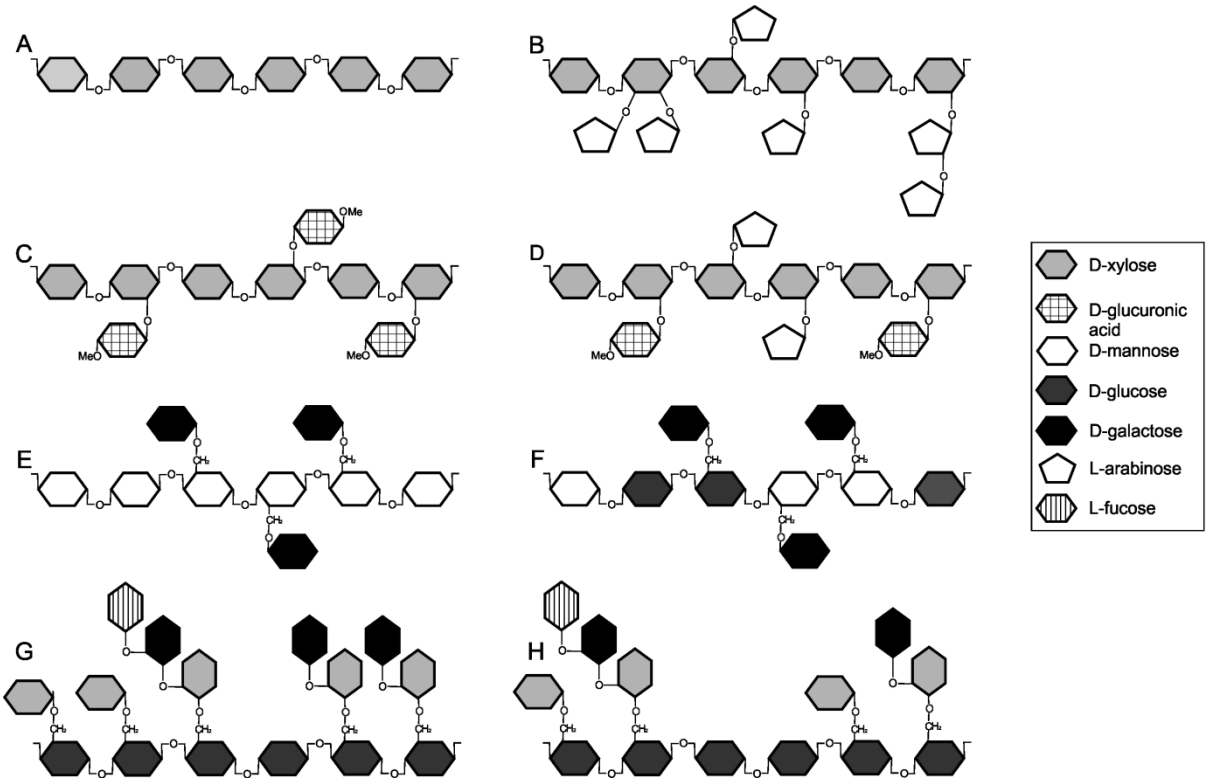

Fig. 1. Examples for structures of common hemicelluloses. A Linear xylan molecule; B Arabinoxylan; C Glucuronoxylan; D Glucuronoarabinoxylan; E Galactomannan; F Galactoglucomannan; G Xyloglucan type XXXG; H Xyloglucan type XXGG;

The three substituted D-glucoses are followed by an unsubstituted one. In type XXGG always two mono-, di- or trisaccharide carrying D-xylose elements alternate with two unsubstituted ones. Xyloglucans present in plant seeds mostly lack substitutions with exception of acetyl

groups (Vincken *et al.* 1997). All types of hemicelluloses are extensively acetylated leading to enhanced hydrophilicity of the molecule and hinder enzymatic degradation but the detailed molecular functions of these O-linked substitutions still have to be elucidated (Bacon *et al.* 1975; Biely *et al.* 1986).

Phenolic acid residues especially ferulic acid and to a minor extend ρ-coumaryl and cinnamic acid are bond to hemicellulose compounds via ester linkages. In addition interconnections to lignin via ether bonds enable the formation of rigid macromolecular structures.

Xylanases in *Trichoderma reesei*

T.reesei secretes highly specialized enzymes that in a synergistic manner attack hemicelluloses in order to release oligo-, di- and monosaccharides that can be assimilated. The composition of the enzyme mixture produced by the fungus differs and is adapted on the present types of hemicelluloses that may vary depending on biomass source and degradation stage.

For xylans in a first step endo-β-1,4-xylanases [EC 3.2.1.8], cleave in the backbone of the molecule thus making it more accessible for further enzymatic action. In *T. reesei* three endoacting xylanases have been identified so far. Two of them, XYN1 and XYN2 belong to glycosyl hydrolase families GH11 and share a common β-jelly roll structure (Törrönen *et al.* 1992) Two glutamic residues in the active site (E86, E177) catalyze the cleavage of glycosidic bonds via a retaining double-displacement mechanism (Jeffries 1996; Törrönen and Rouvinen 1997). GH11 members cleave the D-xylose backbone preferably in unsubstituted regions of the xylan releasing products such as xylotriose and longer xylooligosaccharides (Jeffries 1996; Biely *et al.* 1997). Another endo-β-1,4-xylanase of *T. reesei*, XYN3, has been identified as GH10 family member and was shown to be expressed mainly in the overexpression mutant PC-3-7 (Xu *et al.* 1998; Xu *et al.* 2000). GH10 enzymes generally comprise a classical $(\alpha/\beta)_8$ barrel (TIM barrel) fold with the active centre placed in a cleft. The mechanism of action for GH10 xylanases is a retaining one involving two glutamic acid residues as acid/base and nucleophile. In contrast to GH11 xylanases they are less selective regarding the cleaving sites in the substrate. Actually decorated regions of the xylan are preferred for GH10 action (Biely *et al.* 1997). Once smaller subunits have been released from the large polysaccharide chains exo-acting enzymes attach to the reducing end and release

xylose monomers. A further xylanase belonging to the diverse family GH30 has been identified by Saloheimo and co-workers as mainly exo-acting enzyme releasing xylose monomers and smaller oligosaccharides from the reducing end of linear xylan molecules. The enzyme was termed XYN4 (Saloheimo *et al.* 2003). Further studies revealed that the preferred substrate of XYN4 is rhodymenan, a linear polysaccharide composed of β-1,4-β-1,3 connected D-xylose units. Generally substrates of shorter polymerization grade are preferred (Tenkanen *et al.* 2012). GH30 enzymes share a $(\alpha/\beta)_8$ barrel (TIM barrel) structure and cleave xylanolytic bonds via a retaining mechanism.

In contrast to XYN4 the β-xylosidase BXL1 [EC 3.2.1.37] that also releases monomeric D-xylose from the reducing end of both highly polymerized xylan and xylooligosaccharides does not exhibit any endo activity (Poutanen and Puls 1989; Tenkanen *et al.* 1992; Herrmann *et al.* 1995; Margolles-Clark *et al.* 1996; Tenkanen *et al.* 2012).

Branches and susbstituents attached to xylan are released by specialized side-chain cleaving enzymes. Simultaneously to xylan, arabinan side chains are initially attacked by endo-α-1,5-L-arabinosidases [EC 3.2.1.99] that cleave randomly in the molecule while exo-α-1,5L-arabinofuranosidases [EC 3.2.1.55] catalyze the release of terminal L-arabinose monomers. ABFs in *T. reesei* are very diverse in structure and function thus they belong to different GH families (e.g. GH43, GH51, GH54, GH62). Cleavage of glycosidic bonds is exhibited either via retention or inversion (in case of GH43) (Seiboth and Metz 2011).

Acetylations are removed via acetyl xylan esterases. Two well studied esterases purified from *T. reesei* are AXE1 and AXE2 (Sundberg and Poutanen 1991). The removal of phenolic acids requires specialized esterases. Feruloyl esterases have been purified from *A. niger* and some other fungal species but none yet from *T. reesei*. These enzymes generally show activity with a broad range of substrates such as further phenolic acids (*e.g*. *p*-coumaric acid) and acetyl groups (Tenkanen *et al.* 1991; Faulds and Williamson 1993; Benoit *et al.* 2008).

Polysaccharide uptake

The uptake of mono- and disaccharides into the fungal cell is mediated by specialized transmembrane permease proteins that belong to the major facilitator superfamily, MFS (Pao *et al.* 1998). Regarding substrate specificity high and low affinity transporters are distinguished. In general low affinity transporters are expressed when the substrate is

present at concentrations of around in the mM range. In contrast high affinity transporters are produced by the cell mainly when the concentration of substrate lies in the µM range (Kubicek 2012b). In addition their expression can be repressed by carbon catabolite repression during growth e.g. on glucose. Monosaccharide transporters in yeast share a conserved structure of twelve transmembrane α-helices. A total of twenty hexose transporters and transport related proteins that belong to the yeast hexose transporter family have been identified in *Saccharomyces cerevisae*. The majority of these enzymes also display low affinity to other hexoses such as mannose or fructose and also pentoses (*e.g.* xylose) (Boles and Hollenberg 1997). The main hexose uptake mechanism is facilitated diffusion. Disaccharide sugars such as maltose are imported into the cell using proton symport (Lagunas 1993). For filamentous fungi only a few glucose transporter genes have been characterized so far. To date detailed knowledge about specific L-arabinose, D-galactose or acid transporters in multicellular fungi is still absent (Kubicek 2012a). The expression of these transporters is assumed to be linked to the complex regulatory network of hemicellulase and cellulase gene expression. Thus absence of the carbon catabolite repressor CreA in *A. nidulans* led to derepression of high affinity transporter expression when glucose was present (MacCabe *et al.* 2003). In addition environmental pH has been shown to influence induction of transporter gene expression (Delgado-Jarana *et al.* 2003; van Kuyk *et al.* 2004). The exact mechanisms of how sugar sensing and transporter gene induction are linked are not known yet.

In 2007 Saloheimo and co-workers identified the xylose transporter XLT1 in *T. reesei* by screening a cDNA library for clones that enhance growth on xylose. Heterologous expression of the protein in *S. cerevisiae* led to D-xylose uptake from the liquid medium (Saloheimo *et al.* 2007).

Previous discoveries confirm that there are specific transporters for di- and oligosaccharides in filamentous fungi. In *T. reesei* a β-glucoside permease and a lactose transporter have been identified that were shown to be highly expressed under cellulase inducing conditions (Kubicek *et al.* 1993; Ivanova *et al.* 2013). In addition Porciuncula *et al.* recently identified two specific lactose transporters in the *T. reesei* cellulase overexpression strain PC-3-7. Loss of these proteins led to decreased lactose uptake and cellulase production (Porciuncula Jde *et al.* 2013).

Pentose and hexose catabolic pathways in filamentous fungi

After uptake and transport of the monosaccharides into the cell they are catabolised into smaller units to enter metabolism and are used for generation of energy and built up of new cell compounds. These degradation pathways have been extensively studied in the filamentous fungi *T. reesei*, *A. niger* and *A. nidulans*. An oxido-reductive pentose degradation pathway is present in all these fungi as the main way to decompose D-xylose and L-arabinose into D-xylulose-5-phosphate that enters the pentose phosphate pathway. In an initial NADPH consuming step D-xylose is converted into its corresponding polyol xylitol by a D-xylose reductase [EC 1.1.1.21]. In *T. reesei* and *A. niger* the reductases XYL1 and XyrA, respectively, catalyze this reaction (Hasper *et al.* 2000; Seiboth *et al.* 2003). Xylitol is reduced to D-xylulose in an NAD^+ dependent reaction catalyzed by the xylitol dehydrogenase [EC 1.1.1.12] XDH1 in *T. reesei* and its homolgue XdhA in *A. niger* (Richard *et al.* 2001; Seiboth *et al.* 2003; de Groot *et al.* 2007)

The reduction of L-arabinose in *T. reesei* again requires XYL1 while *A. niger* uses a specific L-arabinose reductase LarA [EC 1.1.1.21] (Seiboth *et al.* 2003; Mojzita *et al.* 2010a) resulting L-arabitol is converted to L-xylulose by the L-arabitol dehydrogenase [1.1.1.12] LAD1 (*T. reesei*) and LadA (*A. niger*) respectively (Richard *et al.* 2001; Pail *et al.* 2004). L-xylulose serves as substrate for the L-xylulose reductases [EC 1.1.1.10] which was part of this work. The resulting xylitol follows the same route as for D-xylose and is converted by XDH1/XdhA to D-xylulose. The pentose gets phosphorylated at position 5 by xylulokinase [EC 2.7.1.17]. XKI1 in *T. reesei* and XkiA of *A. niger* and enters the pentose phosphate pathway, a crucial pathway for regeneration of NADPH and generation of ribose-5-phosphate and erythrose-4-phosphate that are used for nucleotide and nucleic acid synthesis respectively. The presence of an oxido-reductive pathway for pentose catabolism is conserved throughout filamentous fungi. An exception seems to be the anaerobic fungus *Pyromyces sp.* E2 strain which like numerous bacteria expresses a D-xylose isomerase that is used for direct conversion of D-xylose to D-xylulose which is further phosphorylated by xylulokinase (Harhangi *et al.* 2003). There are further D-xylose degradation pathways present in prokaryotes that involve a xylose dehydrogenase for an initial D-xylose oxidation step (Stephens *et al.* 2007). Interestingly a D-xylose dehydrogenase was characterized in *T. reesei* as well but its function remains unclear (Berghäll *et al.* 2007).

More differences between fungal species are found regarding the catabolism of galactose. The most common way to degrade D-galactose is the Leloir pathway. A galactokinase [EC 2.7.1.6] catalyzes the ATP consuming phosphorylation of D-galactose on position 1. Uridylyl addition by the Gal-1-P-uridylyl transferase [EC 2.7.7.12] and subsequent epimerisation to UDP-glucose through UDP-galactose-4-epimerase [EC 5.1.3.2] are further crucial steps of the Leloir pathway. Phosphoglucomutase [EC 5.4.2.2] transfers the phosphate from position 1 to position 6 of glucose. D-glucose-6-phosphate enters glycolysis (Seiboth *et al.* 2002a; Seiboth *et al.* 2002b; Holden *et al.* 2003; Seiboth *et al.* 2004). The Leloir pathway is present in both eukaryotes and prokaryotes. Nonetheless alternative pathways of D-galactose catabolism have been identified in bacteria and fungi. The D-tagatose-6-phophate pathway that has been identified in several bacterial species such as *Staphylococcus aureus* involves an isomerase, kinase and aldolase and thus resembles the Embden-Meyerhof pathway of D-glucose-6-phosphate catabolism (Bissett and Anderson 1980). In filamentous fungi an alternative oxidoreductive pathway of D-galactose degradation has been identified (Fekete *et al.* 2004; Seiboth *et al.* 2004) In nature D-galactose occurs as racemate of α- and β-D-galactose anomers. Only the α-D-galactose can be catabolised via the Leloir pathway directly. The mutarotation of the β-D-galactose anomer is enzymatically driven by an aldose 1-epimerase [EC 5.1.3.3] that is present in many fungi but absent in *T. reesei*, requiring an additional alternative pathway for efficient β-D-galactose catabolism. The enzymes involved in the pathway differ significantly in the fungal species investigated. In general α- and β-D-galactose are converted to galactitol via a reductase [EC 1.1.1.21]. In *T. reesei* this step is catalyzed by XYL1, the same enzyme that is used for D-xylose and L-arabinose reduction (Seiboth *et al.* 2007a). In *A. niger* however a D-galactose reductase, XyrA, is present (Mojzita *et al.* 2012b). In both cases NADPH serves as reducing equivalent. Further L-xylo-3-hexulose is produced from galactitol by LAD1 [EC 1.1.1.12] in *T. reesei* but LadB in *A. niger*, using NAD^+ (Mojzita *et al.* 2012b). The enzymes that catalyzes the reduction of L-xylo-3-hexulose to D-sorbitol utilizing NADPH, the short-chain L-xylo-3-hexulose reductases LXR4 (*T. reesei*) and XhrA (*A. niger*) [EC 1.1.1.10], have been identified in the course of this study. Subsequently, D-sorbitol is oxidized to D-fructose by XDH1 in *T. reesei* and by SdhA in *A. niger* [EC 1.1.1.9] with NAD^+ as cofactor (Koivistoinen *et al.* 2012). Phosphorylation at position 6 via hexokinase HXK1 [EC 2.7.1.1.] generates D-fructose-6-phosphate that enters the pathway of glycolysis (Mojzita *et al.* 2012a).

Regulation of cellulase and hemicellulase expression

In filamentous fungi a complex regulatory network is present that controls the expression of cellulase and hemicellulase genes. The presence of substrates that require the production of (hemi)cellulolytic enzymes by the cell is sensed and communicated over a signalling system involving wide range regulators that still has to be extensively investigated. The binuclear zinc finger transcription factor XYR1 acts as major positive regulator of the cellulolytic and xylanolytic enzymatic system in *T. reesei*. The main cellulases *cbh1, cbh2*, β-galactosidase *bga1,* xylanases *xyn1* and *xyn2*, β-xylosidase *bxl1* and the α-L-arabinofuranosidase *abf2* are controlled by XYR1 (Stricker *et al.* 2006; Akel *et al.* 2009). Direct XYR1 binding to the specific motif $GGC(T/A)_4$ present in promoters of xylanolytic genes mediates their expression (Rauscher *et al.* 2006). In addition loss of XYR1 leads to downregulation of *xyl1*, affecting the catabolic pathways of D-xylose and L-arabinose and thus indirectly regulates the *xyl1* dependent α-L-arabinofuranosidases *abf1* and *abf3* (Stricker *et al.* 2006; Akel *et al.* 2009). The existence of a comprehensive activator is conserved among filamentous fungi. Examples for XYR1 orthologues are XlnR in *A. niger* (van Peij *et al.* 1998; Hasper *et al.* 2000) and *A. nidulans,* AoXlnR in *A. oryzae* (Marui *et al.* 2002) or XlnR in *F. oxysporum* (Calero-Nieto *et al.* 2007). Interestingly the XYR1 homologue of *N. crassa* is limited to the regulation of hemicellulase expression. Cellulases are induced via the zinc binuclear cluster transcription factors Clr-1 and Clr-2 (Coradetti *et al.* 2012). Homologues have been identified in diverse filamentous fungi but their function differs depending on the species. Thus in *A. nidulans* the Clr-2 homologue ClrB is also essential for cellulase production but in contrast to *N. crassa* does not directly induce cellulase expression but is supposed to require a mediator (Coradetti *et al.* 2013). While XlnR mediates D-xylose induced pentose catabolic pathway gene expression another regulator, AraR, specific for the induction of arabinan and L-arabinose catabolism related genes is present in the order *Eurotiales* (Battaglia *et al.* 2011a; Battaglia *et al.* 2011b).

Another positive regulator that was found to trigger the expression of *xyn2* in dependence of the inducing substance is ACE2. Deletion of *ace2* led to a decrease of *xyn2* expression on cellulose but not on sophorose in *T. reesei.* ACE2 responds to the same motif as XYR1 in the *xyn2* promoter and initiates and enables a continuous expression of the protein under influence of distinct inducing signals (Stricker *et al.* 2008b).

Negative regulation occurs through CRE1 mediated carbon catabolite repression. The presence of glucose inhibits the utilization of other lower valued carbon sources by repression of cellulase and hemicellulase gene expression. A main focus regarding the investigation of CCR was put on glucose but also other sugars were shown to induce CCR and uptake itself may have a great impact on this regulation mechanism (Portnoy *et al.* 2011). This regulation is provided by binding of CRE1 to the motif 5'-SYGGRG-3' present in CCR regulated promoter regions. Binding of CRE1 to the promoter prevents recruitment of XYR1 followed by inhibition of gene expression (REF).

A second negative regulator of xylanase expression, ACE1, has been identified in various fungal species. Loss of *ace1* leads to increased expression of xylanase genes independent of the inducing substrate (Aro *et al.* 2003). ACE1 was described to compete with XYR1 for the XYR1 binding site in the *xyn1* promoter thus it indirectly inhibits *xyn1* expression by preventing the activator from binding (Rauscher *et al.* 2006).

The emerging investigation of epigenetic phenomenons led to the identification of whole new levels of gene regulation. In *T. reesei* the putative protein methyltransferase LAE1 acts as regulator of gene clusters involved in secondary metabolite synthesis, iron uptake or receptors. In addition Seiboth *et al.* showed LAE1 to be essential for (hemi)cellulase expression. However regulation of (hemi)cellulolytic gene expression does not occur via direct methylation of the respective chromatin regions by LAE1. Still the mechanisms of LAE1 mediated regulation have to be identified. *Lae1* is conserved among filamentous fungi though there are species specific differences in its function (Seiboth *et al.* 2012b; Karimi-Aghcheh *et al.* 2013).

Aim of the thesis

The shortage and decline of traditional energy sources, especially crude oil, in the near future supports the quest for alternative ways to produce energy, building blocks for (bio)chemical synthesis or higher value products. Filamentous fungi especially *T. reesei* are important industrial workhorses due to their ability to produce huge quantities of extracellular enzymes used e.g. for the production of second generation biofuels or higher value chemicals like polyols from plant biomass. For a knowledge based strain development it is necessary to investigate the catabolic pathways for the different monosaccharides derived from plant biomass and investigate how the extracellular enzymes produced are regulated by the different enzymatic steps and their products accumulating in the different catabolic pathways.

One of the main focuses of this thesis, is therefore the identification of missing links in the degradation of hemicelluloytic monosaccharides such as L-arabinose or D-galactose. The later is also found in the industrial carbon source lactose, an inducer for cellulase production. Based on a better knowledge of these catabolic pathways, the effect of the individual steps on inducer formation and enzyme production will be studied. Following a genome wide analysis of xylanase genes in *T. reesei*, the effect of the interruption of L-arabinose or D-xylose catabolism by specific gene deletions will be studied to identify which pentose is able to induce xylanase gene expression and which steps are responsible or dispensable for inducer formation

Chapter 1

L-xylo-3-hexulose reductase is the missing link in the oxido-reductive pathway for D-galactose catabolism in filamentous fungi.

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L-xylo-3-hexulose reductase is the missing link in the oxido-reductive pathway for D-galactose catabolism in filamentous fungi.

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Contribution of the author

Construction of the *lxr4* knock-out strain; purification of LXR2, LXR3 and LXR4 from cultures of transgenic yeast; enrichment and purification of L-xylulose and L-xylo-3-hexulose from transgenic *E. coli*; plate growth tests; enzyme activity determinations;
The enzymatic related part of the work was performed at the VTT – Technical Research Center of Finland, Espoo, Finland.

Abstract

For the catabolism of D-galactose in fungi, apart from the well-established Leloir pathway, the oxido-reductive pathway has been recently identified. In this oxido-reductive pathway D-galactose is converted is the series of NADPH-dependent reductions and NAD-dependent oxidations into D-fructose. The pathway intermediates include galactitol, L-xylo-3-hexulose, and D-sorbitol. We identified the missing link in the pathway, the L-xylo-3-hexulose reductase which catalyses the conversion of L-xylo-3-hexulose to D-sorbitol. In *Trichoderma reesei* (*Hypocrea jecorina*) and *Aspergillus niger* we identified the genes *lxr4* and *xhrA* respectively that encode the L-xylo-3-hexulose reductases. The deletion of the genes resulted in no growth on galactitol and in reduced growth on D-galactose. The LXR4 was heterologously expressed and the purified protein showed high specificity for L-xylo-3-hexulose with K_m = 2.0 ± 0.5 mM and v_{max} = 5.5 ± 1.0 U/mg. We also confirmed that the product of the LXR4 reaction is D-sorbitol.

Introduction

There are several pathways for the catabolism of D-galactose. The most studied is the Leloir pathway, which exists in prokaryotic and eukaryotic microorganisms. In this pathway, D-galactose is phosphorylated, and in the subsequent steps, converted to D-glucose-6-phosphate in a redox-neutral way (Holden *et al.* 2003). The genes of the Leloir pathway and their regulation have been described in yeast and filamentous fungi (Seiboth *et al.* 2007b). An alternative pathway is the oxidative pathway that is sometimes referred to as the De Ley-Doudoroff pathway and was identified in bacteria (De Ley and Doudoroff 1957). In this pathway, D-galactose is first oxidized to D-galactonolactone, which is then hydrolyzed by a lactonase to D-galactonate followed by the removal of a water molecule by a dehydratase to form D-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-D-galactonate).

D-threo-3-Deoxyhexulosonate is then phosphorylated to D-threo-3-deoxyhexulosonate 6-phosphate, which is subsequently split by an aldolase, resulting in pyruvate and D-glyceraldehyde-3-phosphate. In a strain of the mold *Aspergillus niger*, a nonphosphorylated alternative of the De Ley-Doudoroff pathway was described where the D-threo-3-deoxy-hexulosonate is split by an aldolase to pyruvate and D-glyceraldehyde

instead of being phosphorylated (Elshafei and Abdel-Fatah 2001). Another pathway for D-galactose catabolism has also been demonstrated in some filamentous fungi. It was observed that a *Trichoderma reesei* (*Hypocrea jecorina*) strain with a mutation in the galactokinase gene, *gal1*, is still able to catabolize D-galactose, indicating the existence of an alternative to the Leloir pathway. Because galactitol accumulated in this strain, it was suggested that a pathway exists in *T. reesei* where D-galactose is reduced in the first step (Seiboth *et al.* 2004). In *T. reesei*, an aldose reductase, XYL1, was shown to be responsible for the reduction of D-galactose to galactitol as well as for the reduction of D-xylose and L-arabinose (Seiboth *et al.* 2007a). *A. niger* has distinct L-arabinose and D-xylose reductases, LarA and XyrA (Mojzita *et al.* 2010a). XyrA had the highest activity with D-galactose and was suggested to be involved in D-galactose catabolism (Mojzita *et al.* 2012b). It was also observed in *T. reesei* that a double mutant with deletions in *gal1* and *lad1*, coding for galactokinase and L-arabitol- 4-dehydrogenase, respectively, was not able to catabolize D-galactose. This suggested that the L-arabitol-4-dehydrogenase that is induced on L-arabitol (Richard *et al.* 2001) is also part of this alternative reductive pathway. Pail et al. (Pail *et al.* 2004) showed that purified L-arabitol 4-dehydrogenase, the product of *lad1*, was capable of converting galactitol to L-xylo-3-hexulose. In *A. niger*, it was shown that a distinct galactitol dehydrogenase, LadB, exists besides the L-arabitol dehydrogenase, LadA, that produces L-xylo-3-hexulose and that it is induced on D-galactose and galactitol (Mojzita *et al.* 2012b).

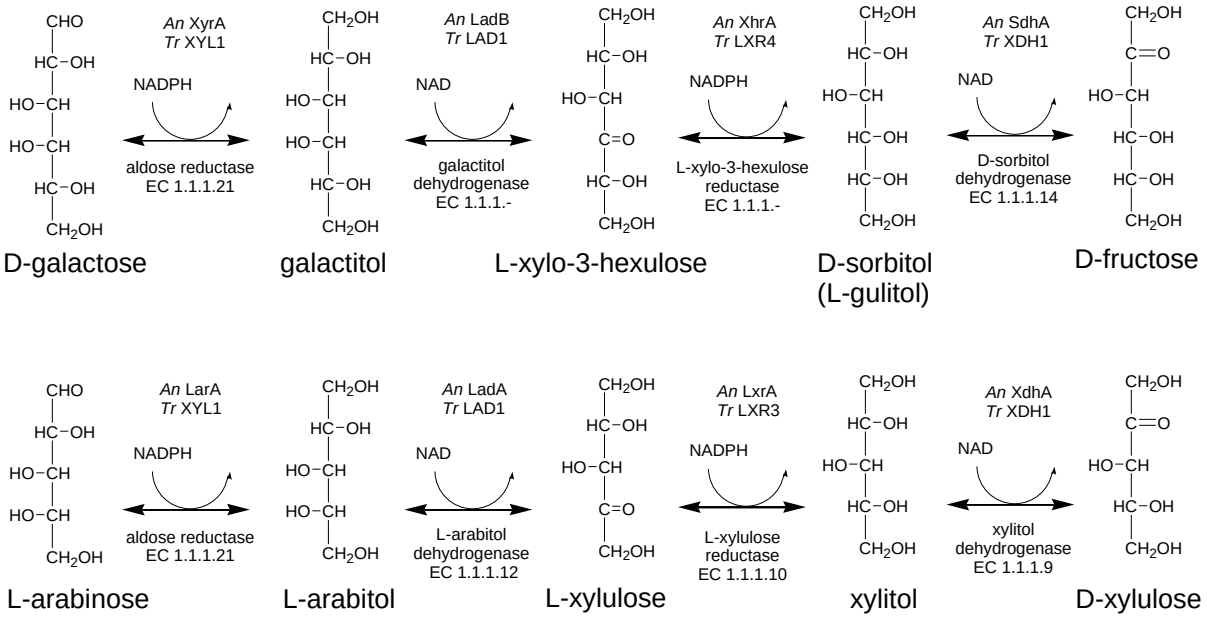

Fig. 1.1. The oxidoreductive D-galactose pathway (*upper part*) and the eukaryotic pathway for L-arabinose catabolism (lower part). The metabolites are in Fischer projection except for the L-*xylo*-3-hexulose and L-xylulose, which are oriented so that the C6 and C5, respectively, are at the *top* to have all molecules in the same orientation. The enzymes for the different reactions in *A. niger* (*An*) and *T. reesei* (*Tr*) are indicated.

Seiboth and Metz (Seiboth and Metz 2011) pointed out the possibility that the L-xylo-3-hexulose is reduced to D-sorbitol in a reaction catalyzed by an enzyme related to L-xylulose reductase or by the L-xylulose reductase itself. The complete oxidoreductive D-galactose pathway would then have the intermediates D-galactose, galactitol, L-xylo-3-hexulose, D-sorbitol, and D-fructose (see Fig. 1.1). D-Sorbitol was shown to be an intermediate in the pathway of *A. niger* where the D-fructose-forming D-sorbitol dehydrogenase, *sdhA*, was found to be induced on D-galactose and galactitol and the *sdhA* deletion mutant had reduced growth on galactitol and was unable to grow on D-sorbitol (Koivistoinen *et al.* 2012). In *T. reesei*, xylitol dehydrogenase, *xdh1*, was proposed to be the enzyme responsible for this reaction. Fekete *et al.* (Fekete *et al.* 2004) suggested that L-sorbose is produced in *Aspergillus nidulans* from galactitol by L-arabitol dehydrogenase and that it could be further converted to D-sorbitol. The reaction mechanism of the galactitol to L-sorbose conversion, however, has not been proposed and currently remains elusive. Here we set out to identify the enzyme that catalyzes the conversion of L-xylo-3-hexulose to D-sorbitol (L-gulitol), which is the missing link in the oxidoreductive D-galactose pathway in the molds *T. reesei* and *A. niger*.

Table. 1.1 The transcription of the following genes was tested in *A. niger* using qPCR. The carbon source was D-galactose or galactitol. The genes can be retrieved from the *Aspergillus niger* homepage at the DOE Joint Genome Institute, JGI (genome.jgi-.doe.gov/Aspni5/Aspni5.home.html) using the JGI identifiers or from the *Aspergillus* genome database (AspGD) using the identifiers in parentheses.

JGI: 177738	(An08g01930)	*lxrA*
JGI: 184209	(An16g01650)	*xhrA*
JGI: 40156	(An07g01830)	
JGI: 174212	(An02g00220)	
JGI: 177858	(An06g01980)	
JGI: 184211	(An16g06440)	
JGI: 56312	(An15g02280)	
JGI: 212729	(An09g00620)	
JGI: 55205	(An04g09990)	
JGI: 212936	(An05g01210)	

JGI: 52907 (An11g02460)

Materials and methods

Strains and chemicals

The *A. niger* strain ATCC 1015 (CBS 113.46) was obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands). The *T. reesei* strain QM9414 (ATCC 26921) was used in this study. For comparison of growth on agar plates, the spores of *A. niger* and *T. reesei* strains were applied to agar plates containing 6.7 g of yeast nitrogen base $liter^{-1}$ (YNB, BD Biosciences), 20 g of agar $liter^{-1}$, and 20 g $liter^{-1}$ of carbon source (see "Results" for details). The L-xylo-3-hexulose used for the *in vitro* tests was produced as described previously (Mojzita *et al.* 2012b).

Transcriptional analysis

The growth of strains, RNA isolation, and qPCR were performed as described previously (Koivistoinen *et al.* 2012).

Deletion of the xhrA *gene in the* A. niger ΔpyrG *strain*

Construction of *A. niger* ATCC 1015 Δ*pyrG* was described previously (Mojzita *et al.* 2010a). The cassette for deletion of the *xhrA* gene (Table 1.1) contained 1644 bp from the *xhrA* promoter region, 1563 bp from the *xhrA* terminator region, and a 1928-bp fragment containing the *pyrG* gene flanked by its native promoter and terminator. These fragments were obtained by PCR from the *A. niger* ATCC 1015 genomic DNA using primers xhrA-5-F, xhrA-5-R, xhrA-3-F, xhrA-3-R, pyrG-del-F_n, and pyrG-del-R_n (Table 2) and the proofreading DNA polymerase Phusion (Finnzymes). The *xhrA* terminator fragment (*xhrA*-3) digested with HindIII (New England Biolabs) was inserted into the plasmid pRSET-A (Invitrogen), which was digested with HindIII and PvuII (both New England Biolabs). This intermediary construct was digested with EcoRV and NheI (both New England Biolabs). The resulting fragment was ligated to the NheI-digested promoter fragment (*xhrA*-5). The resulting vector was digested with EcoRV (New England Biolabs). The *pyrG* DNA fragment, after digestion with SmaI, was inserted between the two *xhrA* flanking regions. The resulting plasmid was verified by restriction analysis and sequencing. The deletion cassette, 5097 bp, containing the *xhrA*

flanking regions and the *pyrG* gene, was released by MluI (New England Biolabs) digestion and transformed into the *A. niger* ATCC 1015 Δ*pyrG* strain. Transformants were selected based on their ability to grow in the absence of uracil. Strains with successful deletions were verified by PCR using the primers xhrA_ORF_F and xhrA_ORF_F (Table 1.2).

Deletion of the lxr4 *gene in* T. reesei

A *T. reesei* Δ*tku70* strain was used as the parental strain for transformation (Gruber *et al.* 1990; Guangtao *et al.* 2009). The cassette for deletion of the *lxr4* gene (GenBankTM accession number BK008566) contained 937 bp of the promoter region, 1162 bp of the terminator region, and a fragment containing the *pyr4* encoding orotidine-5'-phosphate decarboxylase. The promoter and terminator regions were obtained by PCR from the genomic DNA of the *T. reesei* strain QM9414 using primers Trire22771_XbaI-Ups-fw, Trire22771_XhoIUps-rev, Trire22771_XhoI-Dws-fw, and Trire22771_Acc-Dws-rev (Table 1.2) and the proofreading DNA polymerase Phusion (Finnzymes). Both fragments were ligated into the vector pBluescript SK(-) (Stratagene) after digestion with XbaI and XhoI (Fermentas) for the promoter fragment and with XhoI and Acc65I (Fermentas) for the terminator fragment using ligation mix III (Takara). The SalI fragment of *pyr4* served as the selection marker and was cloned via XhiI restriction between the promoter and terminator regions of *lxr4* in pBluescript SK(-). The resulting plasmid (verified by restriction analysis and sequencing) was linearized using XbaI and Acc65I and transformed into the *T. reesei* Δ*tku70* strain. Transformants were selected based on their ability to grow in the absence of uridine. Strains with successful deletions were verified by PCR using the primers dlxr4_for3 and dlxr4_rev3 (Table 1.2).

Reintroduction of xhrA *into the* ΔxhrA *strain*

The *xhrA* gene, with its native promoter and terminator (2496-bp genomic fragment), was amplified from the *A. niger* ATCC 1015 genomic DNA using the primers xhrA-genomic_F and xhrA-genomic_R (Table 1.2). The PCR product was transformed into the Δ*xhrA* strain, and the transformants were selected on medium with galactitol as a sole carbon source. The resulting strains were tested for the presence of the *xhrA* gene by PCR and further analyzed for growth on selected carbon sources.

Expression of the genes in Saccharomyces cerevisiae

For heterologous expression in *S. cerevisiae*, the open reading frames (ORFs) of *xhrA* (Table 1.1) and *lxr4* (GenBank accession number BK008566) were amplified from the cDNA of *A. niger* or *T. reesei*, respectively, grown in the presence of galactitol with the primers xhrA-H-HIS_F, xhrA-ORF_R, lxr4-HIS-N_F, and lxr4_ORF_R (Table 1.2). The genes were inserted into the plasmid pYX212 (Ingenius, R&D Systems, Madison, WI) between the EcoRI and SalI sites (in the case of *xhrA*) or the EcoRI and SmaI sites (in the case of *lxr4*), allowing the expression to be controlled by the *TPI1* promoter. All constructs were verified by sequencing. The *S. cerevisiae* strain CEN.PK2-1D was transformed with the pYX212 plasmids containing the *xhrA* and *lxr4* genes as N-terminal His-tagged variants, and the transformants were selected based on their ability to grow in the absence of uracil. The expression of active reductases was tested in crude cell extracts by enzymatic activity measurements. The *xhrA* was also expressed without a His tag and as a gene that was codonoptimized for expression in *S. cerevisiae*. The gene was also expressed in the *Escherichia coli* strain BL21 (DH3) (Invitrogen) using the pBAT4 expression vector (Peränen *et al.* 1996) and isopropyl-1-thio-_-D-galactopyranoside induction.

Protein extraction, enzyme activity measurements, and analysis of the LXR4 product

For L-*xylo*-3-hexulose-reductase activity measurements in *A. niger* extracts, the parent strain ATCC 1015 and the Δ*xhrA* strains were cultivated overnight in YPG medium (1% yeast extract, 2% Bacto peptone; 3% gelatin). The mycelia were filtered and transferred to fresh medium containing 1% yeast extract, 2% Bacto peptone, and 2% D-glucose, galactitol or L-arabinose and cultivated for 6 h. After incubation in the inducing conditions, the mycelia were isolated by filtration and washed with water, and ~200 mg of wet mycelia were transferred into 2-ml tubes with 0.6 ml of acid washed glass beads (Sigma) and 1 ml of lysis buffer containing 50 mM Tris (pH 7.5) and protease inhibitors (Complete, Roche Applied Science). The cells were disrupted in two 30-s breaking sessions in the 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 analyzed using the Bio-Rad protein assay kit.In the tests performed with the *A. niger* protein extracts, 10 mM L-xylo-3-hexulose, 0.5mM NADPH, and 50 mMTris-HCl (pH 7.5) were used. The enzymatic activity was measured at room temperature by monitoring the NADPH

disappearance at 340 nm in microtiter plates (Nunc) using the Varioskan spectrophotometer (Thermo Electron). For the protein extractions from *S. cerevisiae* and the purification of the His-tagged proteins, the same methods were used as described previously (Mojzita *et al.* 2012b). For LXR4 sugar reductase activities, the reactions were set up in 50 mM Tris-HCl (pH 7.5) with 0.5 mM NADPH and various sugar concentrations (see "Results"). For the LXR4 polyol dehydrogenase activities, the reaction mixtures contained 100 mM Tris-HCl (pH 8.5), 1 mM $NADP^+$, and various concentrations of polyols (see "Results"). The degradation of NADPH or formation of NADPH was monitored as described above. The K_m and V_{max} were estimated from the Michaelis-Menten equation fitted to the measured data. The concentration of L-xylo-3-hexulose produced by *E. coli* expressing *ladB* was determined by NMR spectroscopy. 480 µl of D_2O containing 0.05% of 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP, Aldrich) was added to 120 µl of the sample, and a one-dimensional ^{1}HNMR spectrum was acquired on a 600-MHz Bruker Avance III NMR spectrometer equipped with a QCI CryoProbe (Bruker) using the one-dimensional NOESY pulse sequence for presaturation of the water signal. The concentration of the L-xylo-3-hexulose was obtained by comparing the integral over a region of 4.27–4.40 ppm with the integral of TSP. The *in vitro* reaction with purified LXR4 for the analysis of the reaction product from D-sorbitol was carried out in 1 ml of reaction mix containing 50 µg of the purified LXR4, 100 mM D-sorbitol, and 5 mM $NADP^+$ in 100 mM Tris-HCl (pH 9.0). The reaction mix was incubated at room temperature for 16 h. The control reaction was terminated immediately after the components were mixed by incubation at 95 °C for 10 min. The reactions were analyzed by HPLC as described previously (Mojzita *et al.* 2012b).

Table 1.2 Primers

xhrA-genomic_F	GGCAAGACAACGGACTGAA
xhrA-genomic_R	AATTCCTGGTGATTCGGGTT
xhrA-ORF_F	ATATGAATTCACAATGTCCCTCAAAGGTAAAGTCG
xhrA-ORF_R	ATATGTCGACCTAGATATACAACATCCCACCATT
xhrA-N-HIS_F	ATATGAATTCACAATGCATCACCATCACCATCACGGGTCCCTCAAAGGTAA AGTCG
xhrA-5_F	TATAGCTAGCAGCTGAACGCCTGATACAAA
xhrA-5_R	ATATGATATCGATGGCTTTTGCAGATTGTTTG
xhrA-3_F	ATATGATATCCAGAGCCGTGTTAAATAAGGAATAC
xhrA-3_F	ATTAAAGCTTACGCGTACGAAGCCGCCGAAGATA

act_qPCR_F	CAACATTGTCATGTCTGGTGG
act_qPCR_R	GGAGGAGCAATGATCTTGAC
xhrA_qPCR_F	GATACAGATATGTACCAGGCAG
xhrA_qPCR_R	CTAGATATACAACATCCCACCA
galX_qPCR_F	CTGTGAAATGTTTGGGAAGTC
galX_qPCR_R	GTTTGTTGGTCGTTCGTAGG
pyrG-del-F_n	TATACCCGGGTGATTGAGGTGATTGGCGAT
pyrG-del-R_n	TATACCCGGGTTATCACGCGACGGACAT
lxr4-HIS-N_F	ATATGAATTCACAATGCATCACCATCACCATCACGGGGCCCGTCCGTATGAAGGC
lxr4_ORF_R	TAATGATATCCTACGCAATCGACATGCGCATCC
Trire22771_XbaI-Ups-fw	TCTAGACCATTGTCCCAGCCATCTT
Trire22771_XhoI-Ups-rev	CTCGAGCGACTTGAGCAATCACCAC
Trire22771_XhoI-Dws-fw	CTCGAGGATGTGTACTTGGTGGCTTG
Trire22771_Acc-Dws-rev	GGTACCTCTCTGCTCGTTAAATCCCG
dlxr4_for3	GGCGGAGTTTCTATGGAG
dlxr4_rev3	GGGATTGATATTGTTTGC

Results

Identification of the L-xylo-3-hexulose reductase in A. niger

The suggested oxidoreductive pathway for D-galactose catabolism has similarities with the eukaryotic L-arabinose pathway (Fig. 1.1). The reductions require NADPH, and the oxidations require NAD^+. The reactions that were described are catalyzed by identical or closely related enzymes. In *T. reesei*, the first and second steps of both pathways are catalyzed by the same enzymes. The *T. reesei* XYL1 is the major enzyme for L-arabinose and D-galactose reduction (Seiboth *et al.* 2007a), and LAD1 is the main enzyme for galactitol and L-arabitol oxidation (Richard *et al.* 2001; Pail *et al.* 2004). In *A. niger*, close homologues of different enzymes are used; XyrA is used for D-galactose reduction, and LarA is used for L-arabinose reduction (Mojzita *et al.* 2010a). Also, the second step uses close homologues of different enzymes. Galactitol is oxidized in *A. niger* by LadB (Mojzita *et al.* 2012b), and L-arabitol is oxidized in *A. niger* by LadA (de Groot *et al.* 2007). This information suggested that a similar phenomenon may also exist for the third step. Thus, the L-xylo-3-hexulose reductase may be a homologue of the L-xylulose reductase or identical to the L-xylulose reductase. Because LxrA was identified as the L-xylulose reductase in *A. niger* (Mojzita *et al.* 2010b), we examined

whether this enzyme could also be the L-xylo-3-hexulose reductase. The LxrA was purified using the histidine tag as described previously (Mojzita *et al.* 2010b). The activity was tested with L-xylo-3-hexulose and NADPH and in the reverse direction with D-sorbitol and $NADP^+$. The enzyme had activity with these substrates, suggesting that this enzyme could be the L-xylo-3-hexulose reductase. We tested the transcription of this gene by qPCR during growth on galactitol or D-galactose. The *lxrA* gene was not upregulated under these conditions, suggesting that although the LxrA has L-xylo-3-hexulose reductase activity, it is not the true" L-*xylo*-3-hexulose reductase. Also, the Δ*lxrA* strain does not confer any growth defect in the presence of galactitol when compared with the wild type strain (data not shown). We then tested the transcription of several close homologues to *lxrA* on galactitol or D-galactose, which are listed in Table 1.1. The closest *lxrA* homologue (E-value = 1.45×10^{-31}) JGI: 184209 (An16g01650) was found to be up-regulated on galactitol and D-galactose (Fig. 1.2A). We called this gene *xhrA* for L-xylo-3-hexulose reductase. The *xhrA* gene was also up-regulated on galactitol and D-galactose in a strain where *ladB* was deleted with the up-regulation being significantly higher (Fig. 1.2B). The LadB converts galactitol to L-xylo-3-hexulose, and the *ladB* mutant cannot grow on galactitol (Mojzita *et al.* 2012b). This suggests that galactitol, which probably accumulates in the Δ*ladB* strain and not L-xylo-3-hexulose, is required for the induction of *xhrA*. A homologue of *galX* is located next to *xhrA* on the chromosome. The *galX* gene was identified in *A. nidulans* as the gene encoding a regulator that controls the D-galactose utilization, and it was shown to be up-regulated in the presence of galactitol and D-galactose (Christensen *et al.* 2011). Likewise in *A. niger*, the transcription of *galX* was up-regulated in the presence of galactitol and D-galactose. In addition, *galX* transcription was further enhanced in the Δ*ladB* strain in a similar manner as observed in the case of *xhrA* expression (Fig. 1.2C and D).

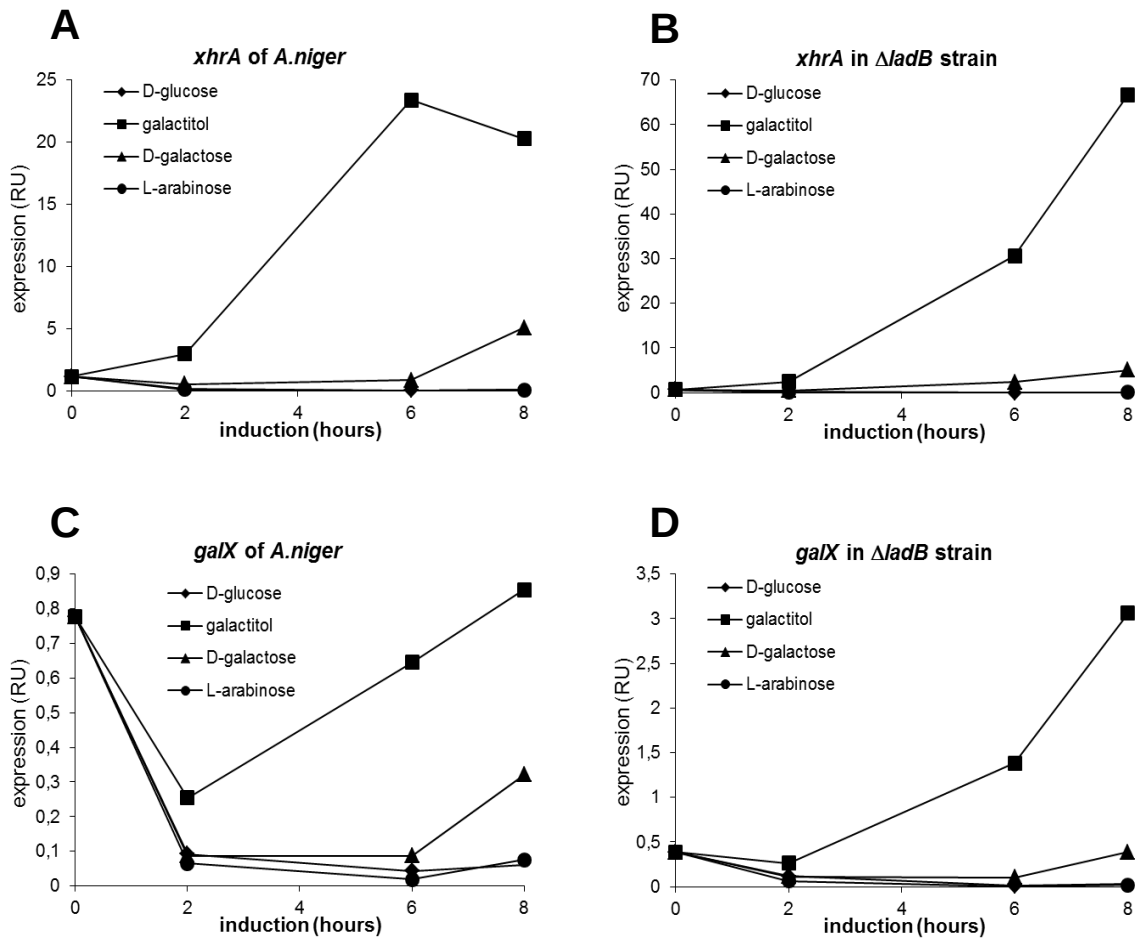

Fig. 1.2. Transcription profiles of the *xhrA* and *galX* genes in *A. niger*. *A*, the *xhrA* gene is up-regulated in the presence of galactitol and to a lesser extent and with a delay on D-galactose. The expression on L-arabinose and D-glucose is not affected. *RU*, response units. *B*, the transcriptional activation of *xhrA* is further increased in the Δ*ladB* strain on galactitol. *C* and *D*, the *galX* encoding the transcription factor responsible for control of the D-galactose catabolic genes is up-regulated on galactitol (*C*), and its transcription is increased in the Δ*ladB* strain (*D*).

Identification of the L-xylo-3-hexulose reductase in T. reesei

In *T. reesei*, several homologues of L-xylulose reductase exist. The product of the *lxr1* gene tre74194 (www.jgi.doe.gov) that was described to be active with L-xylulose (Richard *et al.* 2002) turned out to be a D-mannitol dehydrogenase (Metz *et al.* 2009). Another candidate LXR3 (GenBank accession number BK008567), which was identified and characterized as a gene encoding the true L-xylulose reductase of *T. reesei*, showed only activity with L-xylulose but not with L-xylo-3-hexulose *in vitro* (Metz *et al.* 2012). Moreover, none of these genes are close homologues of *xhrA*. By searching the *T. reesei* genome, we identified a gene that we called *lxr4* (GenBank accession number BK008566) as the closest orthologue for *lxrA*.

Deletion of the L-xylo-3-hexulose reductase genes in A. niger *and* T. reesei

To test whether the *xhrA* is essential for the pathway, we deleted the gene in *A. niger*. The resulting strain was then tested for growth on different carbon sources and compared with the ATCC 1015 parent strain (Fig. 1.3). The mutant showed no growth on galactitol, demonstrating that the *xhrA* is an essential gene for its utilization. To prove that this phenotype is only related to the deletion of the *xhrA*, we retransformed the *xhrA* to the Δ*xhrA* strain. In the strain expressing the *xhrA* in the mutant background, growth on galactitol is fully restored (data not shown). The mutant and parent strain do not grow on D-galactose. Only when small amounts of D-xylose (0.025%) are supplemented does the parent strain grow, and the Δ*xhrA* strain showed reduced growth. 0.025% D-xylose alone does not result in significant growth as described previously (Mojzita *et al.* 2012b). The growth on D-glucose and L-arabinose is not affected because the *xhrA* gene is not required for the metabolism of these sugars. The deletion of *lxr4* in *T. reesei* resulted in no growth on galactitol. The phenotype of this mutant is similar to the *xhrA* deletion in *A. niger* (Fig. 1.3). Growth on galactitol was abolished, but growth on D-glucose and L-arabinose was not affected. Growth on D-galactose is significantly slower but not absent. This is expected because it is established that *T. reesei* has a functional Leloir pathway for D-galactose catabolism and that the oxidoreductive pathway only partially contributes to the D-galactose catabolism. The oxidoreductive pathway only becomes essential if the Leloir pathway is disrupted, for example, when the gene encoding the galactokinase is deleted (Seiboth *et al.* 2004).

Heterologous expression of the L-xylo-3-hexulose reductase

The *xhrA* of *A. niger* and the *lxr4* of *T. reesei* were expressed in *S. cerevisiae* from a multicopy plasmid with a strong constitutive promoter. In the crude *S. cerevisiae* extracts, we could detect L-xylo-3-hexulose reductase activity when the *lxr4* was expressed, but not when the *xhrA* was expressed. We also tested the expression of a yeast codon-optimized version of the *xhrA* gene in yeast as well as the expression of cDNA in *E. coli* using the *lac* promoter and isopropyl-1-thio-β-D-galactopyranoside induction, but we were not able to detect L-xylo-3-hexulose reductase activity (data not shown). Therefore, we used the *T. reesei* enzyme for the *in vitro* characterization of L-xylo-3-hexulose reductase. LXR4 was produced in *S. cerevisiae* as a recombinant N-terminally His6-tagged protein and subsequently purified.

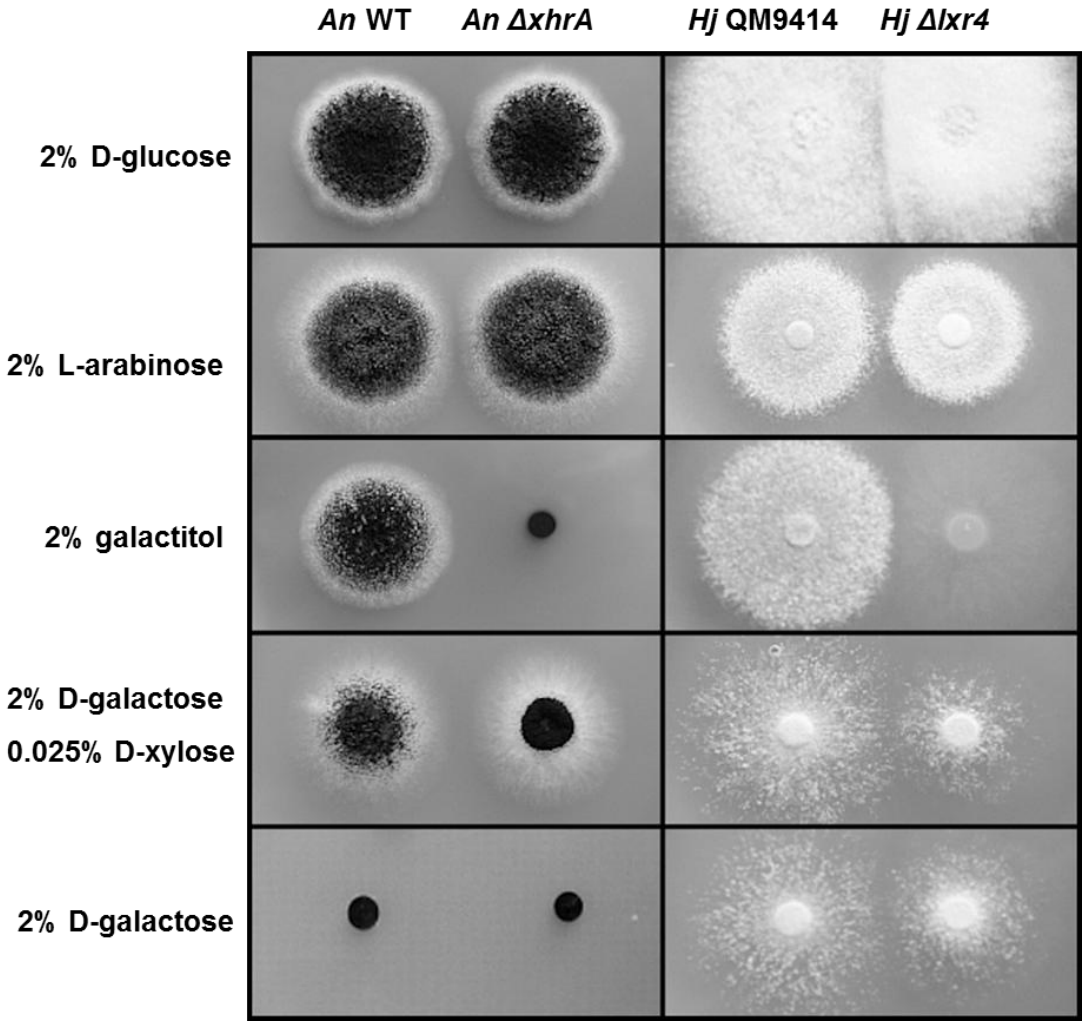

Fig. 1.3. Growth of the *A. niger* (*An*) and *T. reesei* (*Tr*) strains with and without a deletion of the gene encoding L-*xylo*-3-hexulose reductase on solidified medium containing different carbon sources. Equal amounts of spores were applied and grown for 4 days at 28 °C.

The purified enzyme was NADP(H)-specific and conferred high and specific activity with L-xylo-3-hexulose with a K_m = 2.0 ± 0.5 mM and a V_{max} = 5.5 ± 1.0 units/mg (Fig. 1.4*A*). The enzyme was also active with D-ribulose and L-xylulose, K_m = 47 ± 3 mM and V_{max} = 14 ± 2 units/mg and K_m = 22 ± 3 mM and V_{max} = 4.2 ± 1 units/mg, respectively. The enzyme also showed some activity with D-xylulose and very low activity with D-fructose and L- and D-sorbose (Fig. 1.4B and D). In the reverse reaction, LXR4 showed activity with D-sorbitol and D-mannitol, low activity with xylitol, and no activity with galactitol, ribitol, and L- and D-arabitol (Fig. 1.4C and D).

L-xylo-3-hexulose reductase activity in crude extracts of A. niger

Because we were not able to produce an active XhrA in a heterologous host, we tested whether this activity could be detected in *A. niger*. We made a crude cell extract from mycelia that were shifted to different carbon sources. Extracts from mycelia on D-glucose showed the lowest L-xylo-3-hexulose reductase activity. The activity was significantly higher on galactitol and L-arabinose, which is expected because *xhrA* and *lxrA*, respectively, are up-regulated under these conditions. In the *xhrA* deletion mutant, the activity is not increased on galactitol, indicating that the XhrA is mainly contributing to the L-*xylo*-3-hexulose reductase activity under these conditions. On L-arabinose, the activity is not decreased by the *xhrA* deletion. This activity is likely due to *lxrA*, which is up-regulated on L-arabinose and also has high L-*xylo*-3-hexulose reductase activity (Table 1.3).

Table 1.3. L-xylo-3-hexulose activity in crude extracts of *A. niger*. The activities of the crude extract are given in mU/mg of extracted protein. The mycelia were pre-grown in YPG medium and then shifted for 6 hours to YP medium supplemented with 2% carbon sources indicated.

	***A. niger* (WT)**	***A. niger* Δ*xhrA***
D-glucose	7 ± 0.2	7 ± 0.8
galactitol	25 ± 0.8	9 ± 1.0
L-arabinose	42 ± 3.4	60 ± 2.3

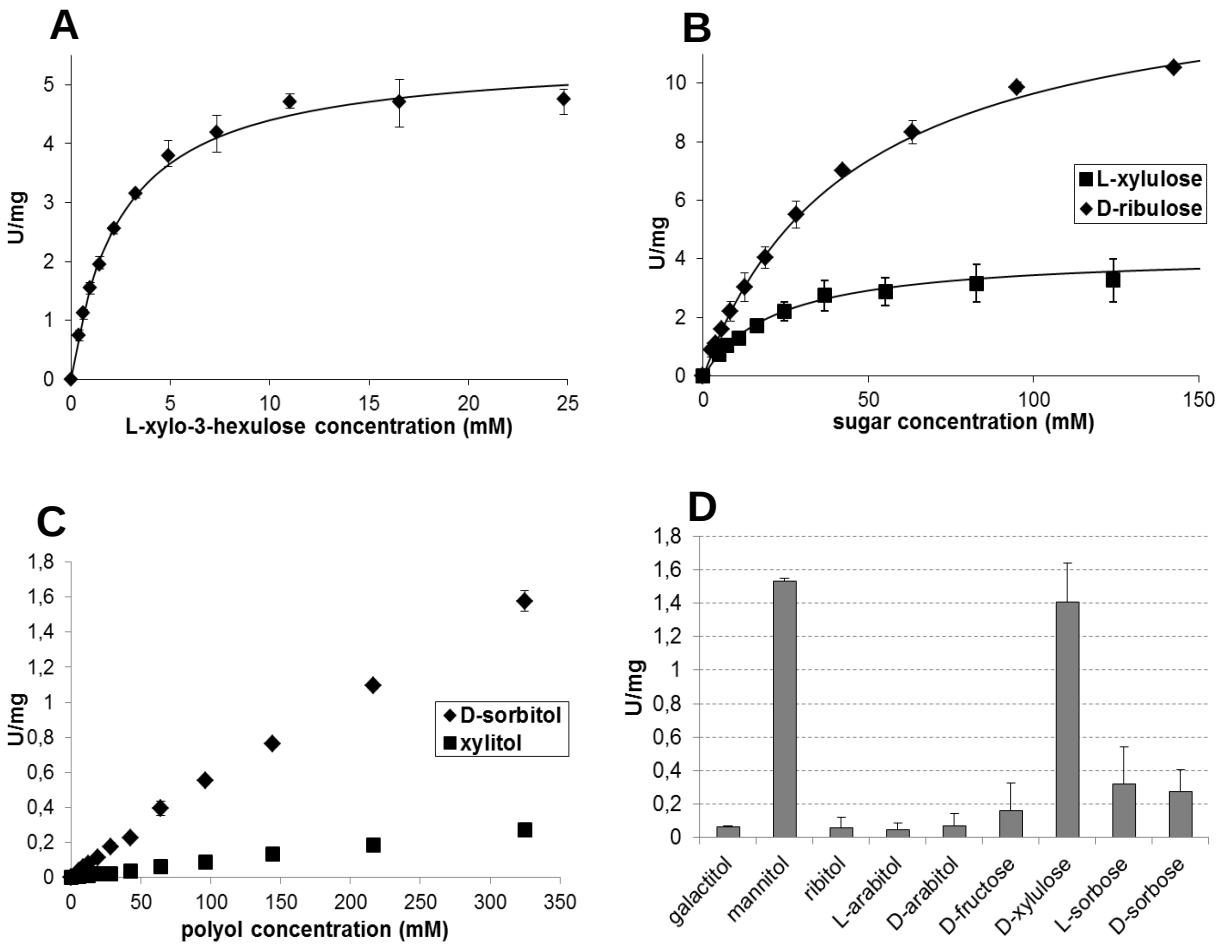

Fig. 1.4. *In vitro* activity of purified LXR4. *A*, initial reaction rate at different L-xylo-3-hexulose concentrations to obtain the Michaelis-Menten-constants: K_m = 2.0 ± 0.5 mM and V_{max} = 5.5 ± 1 units/mg. *B*, initial reaction rates of L-xylulose and D-ribulose. The Michaelis-Menten constants are: K_m = 22 ± 3 mM and V_{max} = 4.2 ± 1 units/mg for L-xylulose and K_m = 47 ± 3 mM and V_{max} = 14 ± 2 units/mg for D-ribulose. *C*, the reverse reaction with the polyols D-sorbitol and xylitol. In the concentration range tested, the rate increased linearly with the substrate concentration, so the Michaelis-Menten-constants could not be determined. *D*, activity with other substrates. The reactions were carried out at room temperature at pH = 7.5 with 0.5 mM NADPH and 50 mM sugars unless otherwise specified. In the reverse direction, 300 mM polyols, 1 mM $NADP^+$, and pH = 8.5 were used. *Error bars* in *panels A–D* indicate S.D.

It had the same retention time as the L-xylo-3-hexulose produced from galactitol using LadB (Mojzita *et al.* 2012b) (Fig. 1.5) or LAD1 (not shown). D-fructose has a different retention time and was not produced. The L-xylo-3-hexulose that we had produced from galactitol (Mojzita *et al.* 2012b) still contained large amounts of galactitol, which overlaps the D-sorbitol signal in the HPLC. This made it problematic to test whether in the forward reaction D-sorbitol was indeed produced from L-xylo-3-hexulose.

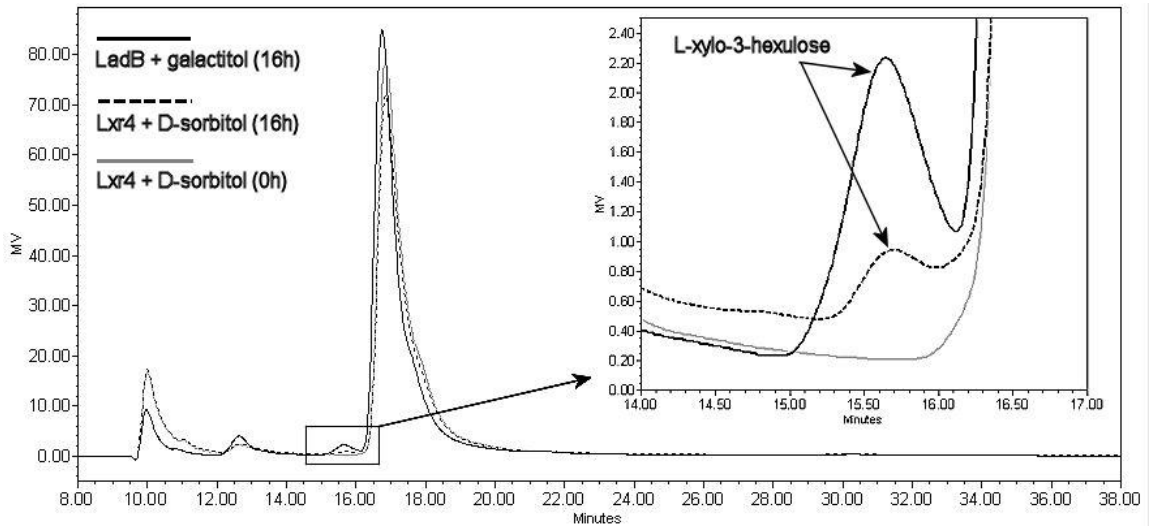

Fig. 1.5. HPLC elution profile of the reaction products of LadB and LXR4. The reaction product formed from galactitol by LadB is L-*xylo*-3-hexulose. The reaction product that is formed from D-sorbitol by LXR4 has the same retention time, which is different from the retention time of D-fructose.

Discussion

In this study, we identified an enzyme with activity for the conversion of L-xylo-3-hexulose to D-sorbitol using NADPH as a cofactor. Such an enzyme activity has not, to the best of our knowledge, been described previously. The enzyme is encoded by *xhrA* in *A. niger* and by *lxr4* in *T. reesei*, and deletion mutants are unable to grow on galactitol. This enzyme activity is the missing link in the oxidoreductive pathway for D-galactose catabolism that had been demonstrated to exist in filamentous fungi. The oxidoreductive D-galactose pathway and the fungal L-arabinose pathway are very similar (Fig. 1.1). In both cases, the sequence of reactions is reduction, oxidation, reduction and oxidation. In both cases, the reductions are NADPH-linked, and the oxidations are NAD^+-linked. In *A. niger*, D-galactose is reduced by XyrA, and L-arabinose is reduced by LarA. In *T. reesei*, the same enzyme, XYL1, is used for the reduction of D-galactose and L-arabinose. In *A. niger*, the second step consisting of the oxidation of galactitol and L-arabitol is carried out by two different enzymes, LadB and LadA, respectively. Again, the same enzyme in *T. reesei*, LAD1, oxidizes both galactitol and L-arabitol. So far, it seems that in *A. niger*, two different pathways exist for D-galactose and L-arabinose, whereas in *T. reesei*, the enzymes of the L-arabinose pathway are also the enzymes of the D-galactose pathway. However, this pattern is different in the third step. In

A. niger, the oxidation of L-xylo-3-hexulose or L-xylulose is performed by two different but highly homologous enzymes, XhrA and LxrA, respectively. However, in this case, *T. reesei* also uses two different enzymes, LXR4 for the L-xylo-3-hexulose reduction and LXR3 for the L-xylulose reduction. The LXR4 has the closest sequence similarity of the *A. niger* enzymes LxrA and XhrA in *T. reesei*. The LXR3, however, is more distant in terms of sequence similarity (supplemental Fig. 1.S1), and it also confers considerably different substrate specificity. For example, it shows no activity with L-xylo-3-hexulose, unlike LxrA, whereas it is active with L-sorbose and D-fructose. In the natural habitats of *T. reesei* and *A. niger*, D-galactose is often accompanied by pentose sugars, which indicates that both L-arabinose and D-galactose pathways are active simultaneously in such conditions. In *T. reesei*, only LXR4 is responsible for the conversion of L-xylo-3-hexulose, whereas in *A. niger*, XhrA and LxrA both contribute to the reaction. Surprisingly, we could not obtain an active XhrA after heterologous expression, although the activity was detected in the *A. niger* crude extracts, and it was reduced in the *xhrA* deletion mutant. That we could not express the active XhrA in a heterologous host could be due to the protein instability, folding problems, or other issues that we did not pursue as we were able to analyze the *T. reesei* homologue (LXR4) *in vitro*.

The last step of the oxidoreductive D-galactose pathway is catalyzed by the D-sorbitol dehydrogenase, SdhA, which was identified in *A. niger*. This enzyme was shown to be part of the pathway because it is up-regulated on D-galactose and galactitol, and the Δ*sdhA* strain showed reduced growth on galactitol (Koivistoinen *et al.* 2012).

In *A. niger*, the corresponding enzyme in the L-arabinose pathway is the xylitol dehydrogenase XdhA (de Groot *et al.* 2007). In *T. reesei*, it is apparently a single enzyme, XDH1, that carries out the sorbitol dehydrogenase reaction and the xylitol dehydrogenase reaction. The enzyme is active with D-sorbitol and xylitol, and the *xdh1* is upregulated on the carbon sources L-arabinose and D-galactose (Seiboth *et al.* 2003). Moreover, the Δ*xdh1* strain fails to grow on galactitol. Although the regulation of the Leloir pathway has been studied and the regulation factors have been identified, not much is known about the regulation of the oxidoreductive pathway. We have suggested previously that galactitol might be the inducing compound for the genes of this pathway (Mojzita *et al.* 2012b). There are four observations that support this. 1) The expression of *ladB* is upregulated sooner and more strongly in the presence of galactitol than in the presence of D-galactose (Mojzita *et al.* 2012b). 2) The upregulation of the *sdhA* is still observed on galactitol in the Δ*ladB* strain

when the pathway is blocked and the production of D-sorbitol, which is the main inducer of *sdhA,* is reduced or even absent (Koivistoinen *et al.* 2012). 3) The expression of the *xhrA* gene is significantly enhanced on galactitol in the Δ*ladB* strain when compared with the wild type strain (Fig. 1.2B). 4) The expression of the *galX* gene, which encodes for the transcription factor involved in the regulation of the Leloir pathways genes, and of the *ladB* gene (Christensen *et al.* 2011) is significantly more expressed on galactitol in the Δ*ladB* strain (Fig. 1.2C). In *A. nidulans*, the D-galactose catabolism and its regulation seem to be different from other filamentous fungi. In this fungus, the use of different D-galactose pathways is pH-dependent. The Leloir pathway is used between pH4.0 and 6.5 and via some other route at pH = 7.5 (Roberts 1970). This other route showed oxidation to galactitol, but the subsequent steps were unidentified. It was suggested that the pathway proceeds via L-sorbose and D-sorbitol or even via sorbose 6-phosphate and D-tagatose 1,6-bisphosphate (Flipphi *et al.* 2009). *A. nidulans* has, in addition to GalX, an additional transcriptional regulator, GalR, which is unique among ascomycetes (Christensen *et al.* 2011). When comparing the different *Aspergillus* species using the comparative analysis tool on the JGI *A. niger* v3.0 database (supplemental Fig. 1.S2), *A. nidulans* does not have a close homologue of *xhrA* in the same location as the other *Aspergillus* species. However, it has a close homologue in a different place. The closest homologue to the *xhrA* is the gene with the identifier ANID_03400.1 (E-value = 2.93×10^{-41}).

Until recently, *A. niger* was considered unable to use D-galactose as a carbon source (de Vries 2008; Fekete *et al.* 2008a). In the latest demonstrations of D-galactose utilization in *A. niger*, the oxidoreductive, but not the Leloir pathway, was shown to be employed, and the D-galactose utilization was enabled with the addition of a small amount of D-xylose (Koivistoinen *et al.* 2012; Mojzita *et al.* 2012b). Fekete *et al. (Fekete et al. 2012a)* recently suggested that the reason for the inability of *A. niger* to germinate on D-galactose is due to nonfunctional D-galactose uptake in the conidiospores, whereas the uptake is active in the mycelium. The authors showed that once the spores germinated on a different carbon source, the mycelium continued to grow on D-galactose; however, it was unable to sporulate. In addition, evidence of a functional Leloir pathway was presented. In the work of Fekete *et al.* (Fekete *et al.* 2012a), the strain N402 (Bos *et al.* 1988) was used. The strain ATCC 1015 that we used in our current and previous studies, nonetheless, behaved differently. The pregrown mycelium was not able to continue growth on D-galactose, but the

strain can sporulate in its presence. In addition, the Leloir pathway is not active, and the growth on D-galactose is facilitated by the addition of a small amount of D-xylose but not D-glucose (Mojzita *et al.* 2012b) (supplemental Fig. 1.S3).

Chapter 2

A novel L-xylulose reductase essential for L-arabinose catabolism in *Trichoderma reesei*.

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A novel L-xylulose reductase essential for L-arabinose catabolism in *Trichoderma reesei*

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Contribution of the author

SH performed growth tests and selected qPCRs; performed L-xylulose production and LXR3 purification with DM; and measured LXR activity of crude extracts with L-xylulose; prepared the final figures for the manuscript, wrote part of the manuscript, read and revised the manuscript.

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 the 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-arabinitol. Deletion of *lxr3* affects growth on L-arabinose and L-arabinitol 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 so far identified true L-xylulose reductases belong to different clades within 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.

Introduction

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 single residue or short side chain in arabinoxylans or as larger branched side chains in the form of arabinan or arabinogalactan in pectins (O'Neill and York 2003; Gilbert 2010). Though the enzymatic steps for the catabolism of L-arabinose were described in the 1960s (Chiang and Knight 1961), most of the genes encoding the enzymes of this five step pathway were only characterized recently (Seiboth and Metz 2011). One reason for this might be that an L-arabinose pathway is not found in the model fungal 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 (Richard *et al.*

2003; Hahn-Hägerdal *et al.* 2007). 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 (Schleif 2000; Watanabe *et al.* 2006). The last two reactions of the fungal L-arabinose pathway are shared with the D-xylose catabolic pathway (Fig. 2.1). 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 (Schleif 2000; Watanabe *et al.* 2006).

Most of the genes and proteins involved in the fungal L-arabinose pathway were characterized in the two ascomycetes *Aspergillus niger* and *Trichoderma reesei* (Seiboth and Metz 2011). 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 (Seiboth *et al.* 2007a; Akel *et al.* 2009). In *A. niger* this NADPH dependend reduction is accomplished by an L-arabinose specific LarA and a D-xylose specific XyrA (Mojzita *et al.* 2010a). The subsequent steps are mediated by L-arabinitol 4 dehydrogenase (Richard *et al.* 2001; Pail *et al.* 2004), L-xylulose reductase (Mojzita *et al.* 2010b), xylitol dehydrogenase (Seiboth *et al.* 2003) and xylulose kinase (van Kuyk *et al.* 2001).

Enzymes with L-xylulose reductase activity are found within the short chain dehydrogenase and reductase family (Kallberg *et al.* 2002) 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 (Pierce *et al.* 2011). The first fungal L-xylulose reductase, ALX1, was identified in the yeast *Ambrosiozyma monospora* and, interestingly, is NADH dependent (Verho *et al.* 2004). Although an enzyme with L-xylulose reductase (LXR1) was described for *T. reesei* (Richard *et al.* 2002), its functional characterization showed that it is actually a D-mannitol 2-dehydrogenase (Metz *et al.* 2009; Metz *et al.* 2011). 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 (Mojzita *et al.* 2010b). 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 (Mojzita *et al.* 2012a).

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

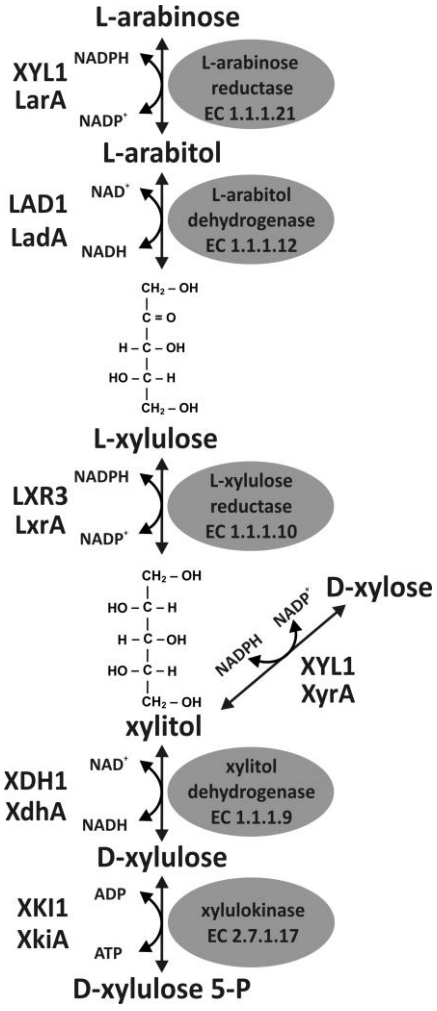

Fig. 2.1. Fungal L-arabinose degrading pathway represented by enzymes of *T. reesei* and *A. niger*. The first three specific steps of the fungal L-arabinose catabolism lead to xylitol, the first common intermediate of the L-arabinose and D-xylose pathway. Xylitol is then converted to D-xylulose 5-phosphate 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.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.

Material and methods

Strains and growth conditions

T. reesei QM9414 (ATCC 26921), Δ*lxr2*, Δ*tku70* (Guangtao *et al.* 2009) and Δ*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 10^6 spores/ml were incubated at 28 °C on a rotary shaker (250 rpm) in 250 ml medium (Mandels and Andreotti 1978) 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 media 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 centre 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 $<10^{-80}$), followed by a BLASTP search to the genome database of the L-arabinose utilizing yeast *Candida guilliermondi*, (http://www.broadinstitute.org/annotation/genome/candida_guilliermondii; e value $<10^{-30}$). 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 under 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 2.1). 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 selection marker (Gruber *et al.* 1990), resulting in pBM1. A 4.9 kb NotI *lxr3* deletion fragment was released from pBM1 and transformed into strain Δ*tku70* (Guangtao *et al.* 2009) as described previously (Gruber *et al.* 1990). For reintroduction of *lxr3,* the pyrithiamine resistance gene *ptrA* of *Aspergillus oryzae* was amplified from vector pME2892 (Krappmann *et al.* 2005) with primers ptrA_fw_PstI and ptrA_rv_HindIII (Table 2.1) and ligated into pBluescript SK(+) (Stratagene). A 2.6 kb DNA fragment containing the whole *lxr3* coding region, ~ 1 kb of the upstream, 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 Δ*lxr3* by electroporation (Schuster *et al.* 2012). 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 (Guangtao *et al.* 2009).

Table 2.1. oligonucleotides used in this study

Oligonucleotide	Sequence
ups-*lxr3-Acc*65I	GGTACCGTCTTCAACTCCTGATAGGG
ups-lxr3-XhoI	CTCGAGGGTCGGAGATCAAGAAAG
dws-*lxr3-Xho*I	CTCGAGCAACAGAAAGAGGTAGACC
dws-lxr3-XbaI	TCTAGACAACTTTAGCACCTGGAGC
RElxr3-Acc65I	GGTACCAACTCCTCGACCGAAATAG
RElxr3-XhoI	CTCGAGTCATGCTCATTGTGTGCTCC
ptrA_fw_PstI	TCTGCAGAAAGCTAGGAGATCGTCC
ptrA_rv_HindIII	TAAGCTTCTCTTGCATCTTTGTTTG
rc_*lxr3*_HisN_fw_EcoRI	ATATGAATTCACAATGCATCACCATC ACCATCACGGGAAGAACGGCGCCTTTCCG
rc_*lxr3*_rv_*Eco*RV	TAATGATATCTCATGGCAGGCTGTAGCCGCC
qPCR_*tef1*_fw	CCACATTGCCTGCAAGTTCGC
qPCR_*tef1*_rv	GTCGGTGAAAGCCTCAACGCAC
qPCR_*xyl1*_fw	AGAACCTGGACAACACCTC
qPCR_*xyl1*_rv	GGCGGAGAAGTAGTTTGTAG

qPCR_*lad1*_fw	GAGCGGTGTCATCGATCTATC
qPCR_*lad1*_rv	TCTTGGGATCTGCTGACGTCTC
qPCR_*lxr3*_fw	AACAGCTCCAAGGCCGCCGTGATTC
qPCR_*lxr3*_rv	AGACACGGTGTTGACGCGGGCAAAG
qPCR_*xdh1*_fw	GCATCTCGGCTGAGGACAAC
qPCR_*xdh1*_rv	CGTGAATGCTCGTCTGGATC
qPCR_*lxr2*_fw	GCCGATATTGGAACAGACG
qPCR_*lxr2*_rv	GAAGACTGCGCCAATGTAC
qPCR_tre122079_fw	TCCAAGGCTGGTGTCATGC
qPCR_tre122079_rv	ATCCAGGCGAGAGTGTGTTG

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 (Chomczynski and Sacchi 1987), 5 µg of the 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 2.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 µl of the 1:10 diluted cDNA (approximately 2.5 ng), 12.5 µl of the iQ SYBR Green Supermix (Bio-Rad), primers (Table 2.1, final concentration of 100 nM) and nuclease-free water in a final volume of 25 µl. 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 the optimum 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© (Pfaffl *et al.* 2002). Individual samples were normalized to the expression of *tef1* (translation elongation factor 1α) as described previously (Seidl *et al.* 2006).

Phylogenetic analysis

Phylogenetic analysis was performed using CLUSTALX version 1.8 (Thompson *et al.* 2002) for protein sequence alignment, GENEDOC version 2.6 (Nicholas and McClain 1987) for visual adjustment, and MEGA version 5 (Kumar *et al.* 2008b) for construction of phylogenetic trees. Neighbour 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) was performed as described for *A. niger* LxrA (Mojzita *et al.* 2010a). An *lxr3* cDNA was amplified with primers rc_lxr3_HisN_fw_EcoRI and rc_lxr3_rv_EcoRV (Table 2.1) and cloned in pYX212 (URA3 selection; Ingenius R&D Systems, Madison, WI), allowing expression of *lxr3* under the TPI1 (triosephosphate isomerase) promoter to produce the recombinant LXR3 with an N-terminal His tag. The *lxr3* cDNA was verified by sequencing.

Preparation of T. reesei *cell free extracts*

T. reesei mycelia grown in liquid culture was washed and ground in liquid nitrogen. Per gram of mycelia (wet biomass) 3 ml of extraction buffer was added (PBS) [8g/l NaCl, 0.2g/l KCl, 1.44g/l Na2HPO4, 0.24g/l KH2PO4, (pH 7.4), and 5 mM β-mercaptoethanol] and the mixture homogenized (12 x 20 s, duty cycle 25%, output 2) with a Branson model 250 Sonifier (Germany) 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 Company) 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 2 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 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 mycelium 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; 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 (BioRad).

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 0.2mM 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 as nanokatals and are given as specific activities (nanokatal per milligram of protein). High-performance liquid chromatography measurements were performed as described previously (Akel, Metz et al. 2009; Metz, de Vries et al. 2009). Enzyme measurements for L-arabinose reductase, L-arabinitol dehydrogenase, and xylitol dehydrogenase activity in cell free extracts were described previously (Seiboth, Hartl et al. 2003; Seiboth, Gamauf et al. 2007).

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 mycelia fungi and present in the L-arabinose-utilizing yeast *C. guilliermondi*. 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 2.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 (Fig. 2.2). *lxr3* 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 an lxr2 *and* lxr3 *deletion 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* (Guangtao,

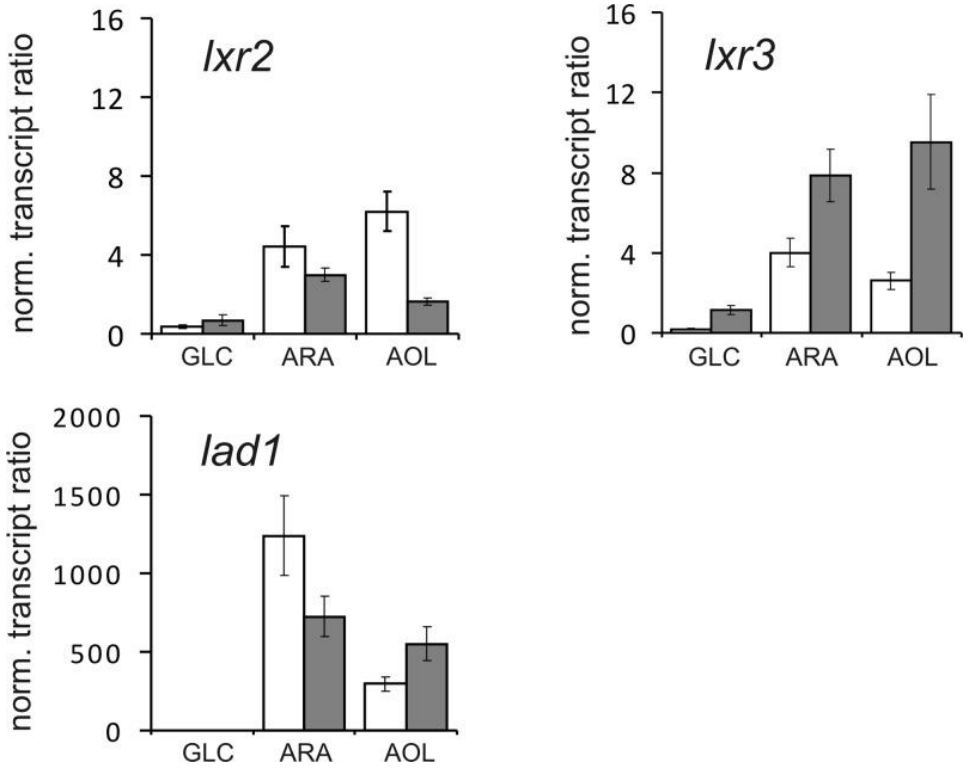

Fig. 2.2. Transcriptional analysis of *T. reesei lxr2* and *lxr3*. *T. reesei* QM9414 was cultivated for 24 h on glycerol and replaced to new media containing 1% of the indicated carbon source (GLC, D-glucose; ARA, L-arabinose, AOL, L-arabinitol) for 2 (dark bars) and 8 hours (grey bars). Expression of the *lxr2, lxr3* and *lad1* is related to their expression on glycerol after 24 h and normalized to the expression of *tef1*.

Hartl et al. 2009) 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 (Fig 2.3A). 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-arabinitol (Fig. 2.3A,B). No effect ,however, was found for ,*e.g.*, D-glucose or D-xylose as the carbon source. A reintroduction of *lxr3* in the Δ*lxr3* strain restored growth on L-arabinose and L-arabinitol (Fig. 2.S1 of the Supporting Information).

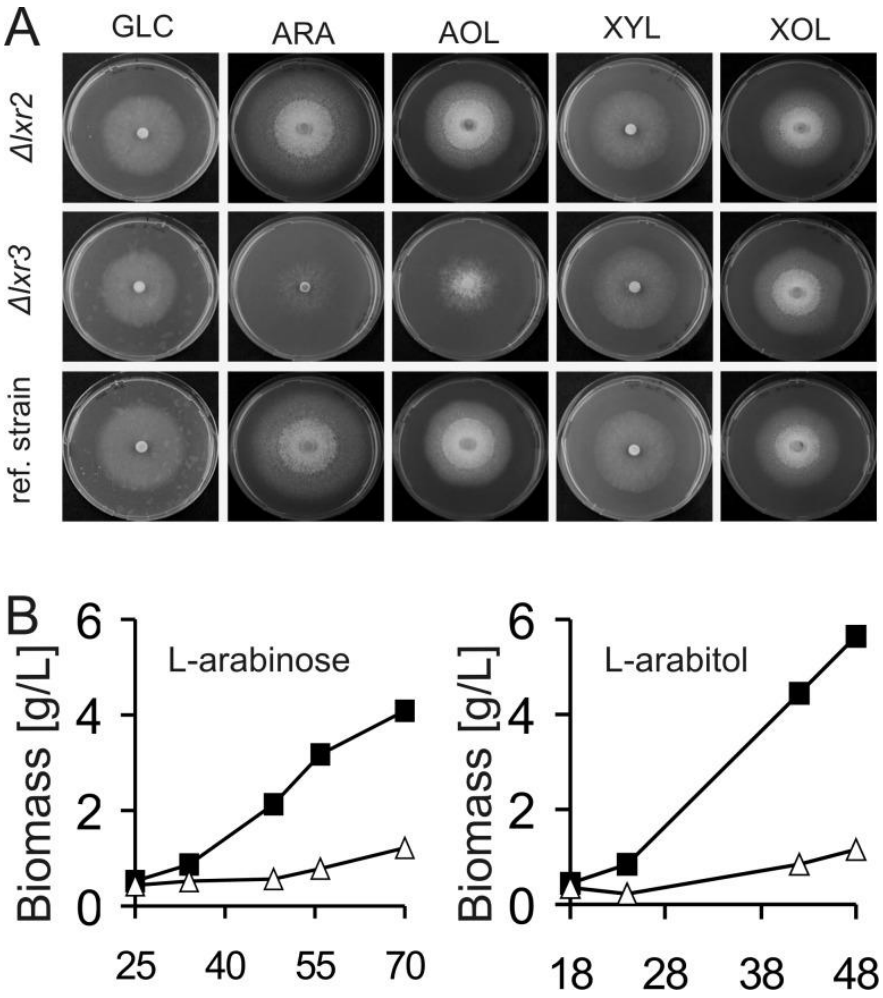

Fig. 2.3. Effect of deletion of *lxr2* and *lxr3* on growth on different carbon sources. (A) Radial growth on agar plates after 3 days and (B) biomass accumulation during liquid cultivation on different carbon sources (1%, w/v) as indicated for *lxr3* (△) compared to the parental strain (■): GLC, D-glucose; ARA, L-arabinose, AOL, L-arabinitol; XYL, D-xylose; XOL, xylitol.

Deletion of lxr3 *affects the total L-xylulose activity and the regulation of L-arabinose metabolism*

The prominent effect of the *lxr3* 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 Δ*lxr3* strain. LArabinose- 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 *lxr3* led to a significant reduction in NADPH specific LXR activity after replacement to both media containing L-arabinose (Fig. 2.4), while NADH specific LXR activity remained constant (*e.g.*, 0.6 nkat/mg on L-arabinose containing minimal medium). Again, the deletion of *lxr2* had no negative influence on LXR

activity, indicating that LXR3 is responsible for the major NADPH specific L-xylulose reductase in *T. reesei* during growth on L-arabinose as the carbon source. To examine if a deletion of *lxr3* 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 Δ*lxr3* 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 (Fig. 2.5A). This change 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 (Fig. 2.5B).

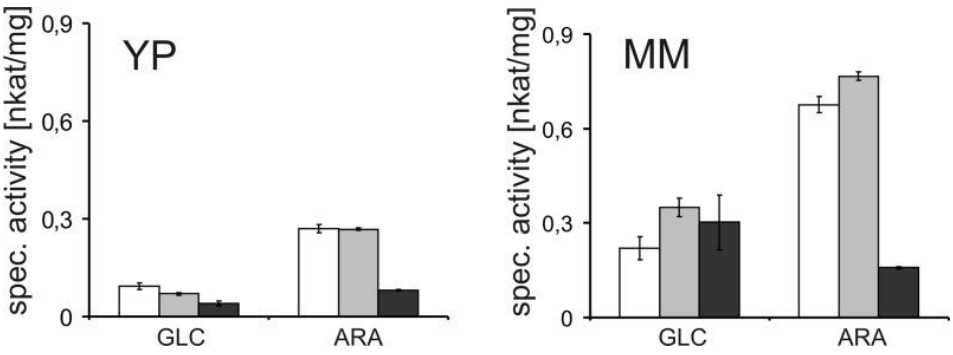

Fig. 2.4. Effect of the deletion of *lxr2* and *lxr3* on total L-xylulose reductase activities. Mycelia were pregrown before the medium was replaced with rich (YP) or minimal medium (MM) containing either 1 % (w/v) D-glucose or L-arabinose for 6 or 15 h. NADPH dependent LXR activity was tested in crude protein extracts for QM9414 (white bars), Δ*lxr2* (grey bars), and Δ*lxr3* (dark grey bars).

Characterization of the 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 (Mojzita *et al.* 2012a) 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.

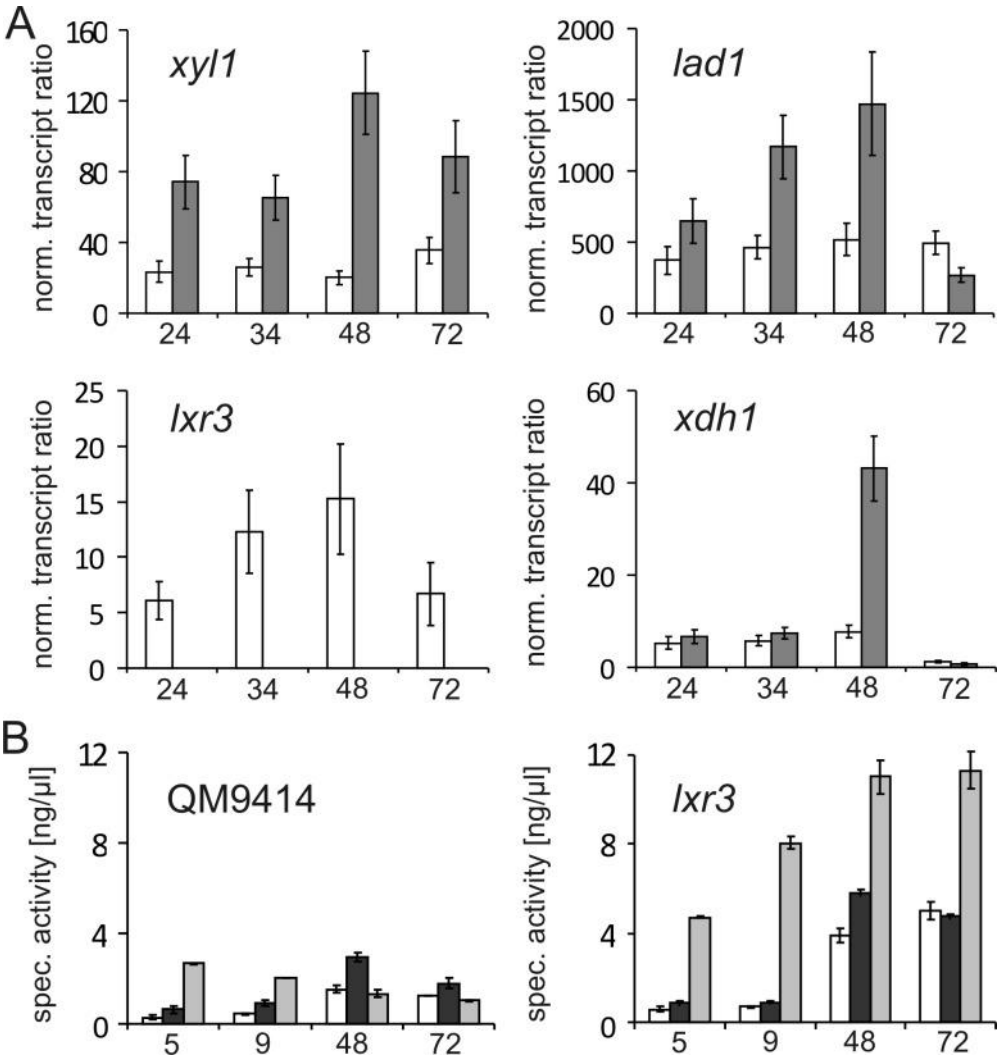

Fig. 2.5. Consequences of the deletion of *lxr3* on the expression of other genes of the L-arabinose pathway. (A) Transcript levels of *xyl1*, *lad1*, *xdh1* and *lxr3* relative to the expression during growth on glycerol at 24 h and normalized to *tef1*. (B) Total L-arabinose reductase (white bars), L-arabinitol dehydrogenase (dark grey bars) and xylitol dehydrogenase activity (light grey bars) were measured in QM9414 and Δ*lxr3.* Strains were either precultivated on glycerol and replaced with L-arabinose (5 and 9 h) or directly cultivated on L-arabinose (48 and 72 h).

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-xylo-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 (Fig. 2.6 and Fig. 2.S2 of the 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-xylo-3-hexulose reductase LXR4, another that contained LXR1, and a third that was split into two subclades containing LXR2 and LXR3.

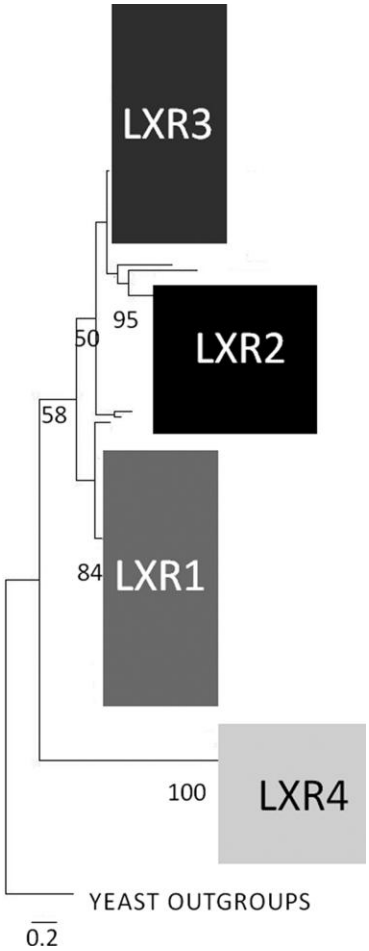

Fig. 2.6. Scheme of the phylogenetic relationship of *T. reesei* LXR3 to other in vivo functional L-xylulose reductases, including the L-xylulose reductase LxrA of *A. niger* and ALX1 of *Am. monospora* found in yeast outgroups. Also included are LXR1 (D-mannitol 2-dehydrogenase, which also exhibits L-xylulose reductase activity), LXR2, and the L-xylo- 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 Fig. 2.S2 of the Supporting Information.

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 Lxylulose 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 also the D-xylose pathway have been characterized from the two ascomycetes *A. niger* and *T. reesei*. Although the overall sequence of reactions is conserved consisting of four oxidoreductive steps and a final phosphorylation (Fig. 2.1), 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-xylose reduction in *T. reesei*, but two rather specific reductases for L-arabinose (LarA) and D-xylose (XyrA) in *A. niger* (Seiboth *et al.* 2007a; Mojzita *et al.* 2010a). Here we identified 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 (Pierce *et al.* 2011). 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* (Verho *et al.* 2004) and the recently identified NADPH-dependent LxrA of *A. niger* (Mojzita *et al.* 2010b). Although it was assumed for many years that the LXR step is NADPH-dependent (Chiang and Knight 1961; Witteveen *et al.* 1989) 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 *lxr3* 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 (Witteveen *et al.* 1989; Mojzita *et al.* 2010b). 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 nkat/mg) (Mojzita *et al.* 2010b) but higher than that of purified LXR1 (75 nkat/mg) (Richard *et al.* 2002).

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-xylo-3-hexulose whereas LXR4 is able to do so (Mojzita *et al.* 2012a). 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-xylo-3-hexulose, the product of LAD (Pail *et al.* 2004; Gilbert 2010) 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-xylo-3-hexulose.

We have recently identified yet another SDR LXR4 that is involved in this step in oxidoreductive D-galactose catabolism (Mojzita *et al.* 2012a). A major consequence of the *lxr3* 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 *lxr3*, *i.e.*, *xyl1* and *lad1*, while *xdh1* that is responsible for the step downstream of *lxr3* is only upregulated to a later

time point. This would imply that the inducer for L-arabinose catabolic genes *xyl1* and *lad1* is produced upstream of *lxr3*, while the inducer for the upregulation of *xdh1* accumulates at a later time point.

Chapter 3

Prepared for the possible: xylanase gene transcription in *Trichoderma reesei* is triggered by different inducers representing different hemicellulosic pentose polymers.

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Prepared for the possible: xylanase gene transcription in *Trichoderma reesei* is triggered by different inducers representing different xylans

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Contribution of the author:

SH performed all experimental work included in this study except GC measurements, prepared the figures for the manuscript, wrote the mainpart of the manuscript, revised the manuscript

Abstract

The ascomycete *Trichoderma reesei* is a paradigm for the regulation and production of plant cell wall degrading enzymes including xylanases. Four xylanases including XYN1 and XYN2 of the glycosyl hydrolase family 11, the GH10 XYN3 and the GH30 XYN4 were already described. By genome mining we identified a fifth xylanase XYN5 belonging to GH11. Transcriptional analysis reveals that the expression of all xylanases but *xyn3* is induced by D-xylose, dependent on the cellulase- and xylanase-regulator XYR1 and negatively regulated by the carbon catabolite repressor CRE1. Impairment of D-xylose catabolism at the D-xylose reductase and xylitol dehydrogenase step strongly enhances induction by D-xylose. Knock out of the L-xylulose reductase encoding gene *lxr3*, which connects the D-xylose and L-arabinose catabolic pathway has no effect on xylanase induction. Beside the induction by D-xylose, the *T. reesei* xylanases are also induced by L-arabinose, and this induction is also enhanced in knock out mutants in L-arabinose reductase (*xyl1*), L-arabitol dehydrogenase (*lad1*) and L-xylulose reductase (*lxr3*). Induction by L-arabinose is also XYR1 dependent. Analysis of intracellular polyols revealed accumulation of xylitol in all strains only during incubation with D-xylose, and accumulation of L-arabitol only during incubation with L-arabinose. Induction by L-arabinose can be further stimulated by addition of D-xylose. We conclude that the expression of the *T. reesei* xylanases can be induced by both D-xylose and L-arabinose, but independently of each other, and by using different inducing metabolites.

Introduction

Current attempts to use plant biomass for production of advanced biofuels and high value chemicals in biorefineries have fortified the interest in the enzymatic hydrolysis of its polysaccharide components. Besides cellulose, hemicelluloses can make up to 30 % of the plant dry material, of which xylan is the major hemicellulose polymer in cereals and hardwood. Xylan consists of a β-1,4-linked D-xylose backbone, to which other residues such as L-arabinose, 4-*O*-methyl-glucuronate, and acetyl side chains can be attached thus resulting in a high variety of xylan structures.

The sordariomycete *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is a paradigm for research on cellulases and hemicellulases, and used as a producer of these enzymes by

various companies (Beg *et al.* 2001; Dashtban *et al.* 2009). With regards to its xylanases, four have been purified and characterized: two members of glycosyl hydrolase family GH11 (XYN1 and XYN2) (Tenkanen *et al.* 1992; Törrönen *et al.* 1992), the GH10 member XYN3 (Xu *et al.* 1998; Ogasawara *et al.* 2006) and the GH30 member XYN4 (Saloheimo *et al.* 2003). While the former three are all endo-β-1,4-xylanases, XYN4 is classified as a xylan 1,4-β-xylosidase, because it produced D-xylose as the main end product from xylan. It also displayed greater activity towards unsubstituted xylans or acetylated methylglucuronic acid-xylans than the GH10 and GH11 xylanases (Saloheimo *et al.* 2003; Tenkanen *et al.* 2012).

The formation of the enzymes needed for degradation of cellulose and hemicelluloses in *T. reesei* is adaptive and occurs only in the presence of an inducer. While the cellulases of *T. reesei* are known to be coordinately regulated by cellulose, lactose and the β-1,2-diglucoside sophorose (Foreman *et al.* 2003; Kubicek *et al.* 2009) differences in the induction of the xylanases were reported: expression of *xyn1* is induced by D-xylose, whereas *xyn2* is induced by xylobiose and the cellulase inducing carbohydrates cellulose and sophorose (Zeilinger *et al.* 1996). *xyn3* expression was found only in a mutant strain (*T. reesei* PC-3-7) and there only induced by cellulase inducers but not D-xylose (Xu *et al.* 2000). No data are available on the expression of *xyn4*. Xylanase expression by D-xylose is regulated via the transcriptional activator XYR1 or its orthologous XlnR and by general carbon catabolite (de)repression in *T. reesei* and other fungi including e.g. *Aspergillus* spp., *Neurospora crassa* and *Fusarium* spp, although species specific adaptions are found (van Peij *et al.* 1998; de Vries *et al.* 1999; Marui *et al.* 2002; Calero-Nieto *et al.* 2007; Stricker *et al.* 2008a; Seiboth *et al.* 2012a; Sun *et al.* 2012). In *T. reesei xyn1* and *xyn2* respond in different ways to carbon catabolite repression (Mach *et al.* 1996).

Triggering of expression of polysaccharide hydrolyzing enzymes is often achieved by different mono- or disaccharides arising from the hydrolysis of the polysaccharide (Kubicek 2012b). However, whether these compounds or metabolites derived from them are the actual inducers is mostly not known. D-xylose is converted via D-xylose reductase, xylitol dehydrogenase and xylulokinase to D-xylulose 5-phosphate to enter the pentose phosphate pathway. The pathway is interconnected to L-arabinose catabolism which involves an L-arabinose reductase, L-arabitol dehydrogenase and L-xylulose reductase to form xylitol, the first common intermediate (cf. Fig. 4, reviewed in (Seiboth *et al.* 2012a)). Mach-Aigner *et al.* (Mach-Aigner *et al.* 2010; Mach-Aigner *et al.* 2011) studied induction of the xylanases *xyn1*

and *xyn2* in *T. reesei* strains blocked in specific steps in the D-xylose and L-arabinose catabolic pathway and concluded that the first step in D-xylose catabolism catalyzed by the D-xylose reductase XYL1 is necessary for (full) induction of *xyn1* and *xyn2* and hypothesized that L-arabitol, formed from D-xylose, would be the true inducer of xylanase expression.

Here we have studied the regulation of all xylanase encoding genes of *T. reesei*, using mutants in the D-xylose and L-arabinose catabolic pathway to obtain a hint towards the inducer of xylanase gene expression. We will show that, in contrast to the above findings, absence of XYL1 enhances induction of the inducible xylanolytic enzyme system (XYN1, XYN2, XYN4 and XYN5) by D-xylose. In addition, we will show that L-arabinose also induces xylanase transcription, but independent of D-xylose.

Materials and methods

Strains and Cultivation Conditions

T. reesei strain QM 9414 (ATCC 26921) (Mandels *et al.* 1971), that served as reference strain, and the knock-out strains Δ*xyl1* (Seiboth *et al.* 2007a), Δ*lad1*, Δ*xdh1*, Δ*lad1*/Δ*xdh1* (Seiboth *et al.* 2003), Δ*lxr3* (Metz *et al.*, submitted), Δ*xyr1* (Stricker *et al.* 2006) and Δ*cre1* (Portnoy *et al.* 2011) were precultured for 24 h in 250 ml Mandels Andreotti medium (MA) (Mandels and Andreotti 1978), containing 1% (w/v) glycerol as sole carbon source, on a rotary shaker (250 rpm) at 28°C. Subsequently mycelia were collected, washed and transferred to 250 ml MA medium without carbon source. After 30 minutes of incubation the inducing carbon source (D-xylose, xylitol or L-arabinose) was added to the cultures. Samples of mycelia were taken directly before adding D-xylose and after 2 h, 4 h and 6 h of induction. As control all strains were cultured in MA without D-xylose.

Quantification of xylanase gene expression.

The mRNA was extracted following a phenol-chloroform based approach (Chomczynski and Sacchi 1987). cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas), following the manufacturer's protocol. Quantitative real time PCR (qPCR) reactions were performed on a BIO-RAD® iQ™ thermal cycler. The reaction mix contained 12.5 µl SYBR® Green Supermix (BIO-RAD®), 8.5 µl pure water (ROTH), 1 µl forward primer (160 mM), 1 µl reverse primer (160 mM) and 2 µl of 1:100 diluted template cDNA.

Oligonucleotides are listed in Supplementary Table 1 except for *xyn1* (Mach-Aigner *et al.* 2010). The *tef1* (encoding transcription elongation factor 1α) gene was used as internal standard. Expression data were evaluated using REST® software (Pfaffl *et al.* 2002). Reactions were performed in triplicates. Data correspond to at least two biological replicas.

Analysis of phylogeny and evolution

DNA and protein sequences were visually aligned by using Genedoc 2.6 (Nicholas and Nicholas Jr 1997). Phylogenetic trees were constructed by the neighbor joining method (Portnoy *et al.* 2011), using the computer program MEGA, version 5.0 (Tamura *et al.* 2011). Unalignable N- and C-terminal regions in the amino acid sequences were omitted from the analyses, and gaps and missing data were pairwise deleted. The pairwise K_a/K_s ratio was determined with DNASp 5.0 (Librado and Rozas 2009). Codon based Fisher's test and the Codon-based Z test implemented in MEGA 4.0 (Tamura *et al.* 2007) were used to directly test the hypotheses of evolutionary models.

Sugar and polyol quantification

Carbohydrates in the medium were analysed by HPLC essentially as described previously (Metz *et al.* 2009) using 5 mM sulfuric acid as eluent at 40°C. For analysis of the intracellular sugars and polyols, mycelia were collected by centrifugation (3 min, 8.000 rpm), washed with ddH_2O and resuspended in 1 ml of ddH_2O. This suspension was heated at 100 °C for 10 min, centrifuged (10 min, 14.000rpm) and the supernatant used for analysis by GC, using D-sorbitol as a standard. Samples were vacuum dried, and then converted to methylsilyl derivates by addition of 50% pyridine, 35 % hexamethyldisilazane and 15% (v/v) trimethylchlorosilane following incubation at room temperature o/n. Samples were measured in an Agilent 7890A GC system using a HP-5 column (l = 30 m, d = 0.32 mm and 0.25 µm film thickness) with an FID detector (Agilent Technologies, Santa Clara, USA). The temperature program was 100°C for 1 min, followed by a temperature increase of 5°C/min to 220°C, an increase of 35°C/min to 320°C, and kept constant at 320°C for 5 min. The helium flow rate was set to 1.4 ml/min, injector temperature 260°C and the detector temperature 300°C. Data are related to an intracellular volume of 2.4 ml per g of dry biomass (Slayman and Tatum 1964).

Results

Identification of a fifth T. reesei *xylanase, XYN5*

In order to study the regulation of the complete xylanolytic system of *T. reesei*, we mined its genome database for eventual yet undescribed xylanases by using a BLASTP search with different fungal xylanases. This led to the identification of indeed one further xylanase gene, *xyn5*, whose deduced protein sequence encoded a third glycosyl hydrolase (GH) family 11 member. XYN5 is annotated incorrectly as a GH18 family member in the *T. reesei* genome homepage (http://genome.jgi.doe.gov/Trire2/Trire2.info.html) and in GenBank (EGR44310). To learn its relationship to the two other GH11 members XYN1 and XYN2, we used it as a query in BLAST search and picked out the 38 best hits (< e-100) for phylogenetic analysis by the maximum likelihood method. As can be seen in Fig. 3.1, the xylanases were grouped into two major clades, one containing *T. reesei* XYN1 and the other XYN2. A small clade between them, containing xylanases from *Myceliophora thermophila* and related proteins, could not be safely aligned with either of these two clades but as this was not relevant to the present investigation it was not further elucidated.

The new XYN5 and its orthologues from *T. virens* and *T. atroviride* formed a sister clade to XYN1 in the respective clade. Interestingly, the XYN1 clade lacked any relationship to the respective species phylogeny, and contained enzymes from several *Penicillium/Talariomyces* spp. It also contained a further clade consisting of *T. virens* and *T. atroviride* GH11 xylanases that were lacking from *T. reesei*. Since the accumulation of paralogs for this gene family could indicate a selective advantage of these genes for *Trichoderma* spp., we examined the evolutionary forces driving these gene duplications: to this end we calculated the K_a/K_s ratio for all pairwise combinations of xylanase exons in the XYN1 clade for all three *Trichoderma* spp. In all cases we only obtained ratios that were significantly less than 1, with a mean Tajima's D of 0.254, implying that nucleotide sequence differences between genes have primarily occurred at synonymous sites. Plotting K_S vs. K_a showed that the K_S values for some gene-to-gene comparisons are very high (up to 1.8) and have apparently reached the saturation level (Nei and Kumar 2000). These finding suggest that the XYN1 clade evolves by purifying selection.

In order to test for this directly, we used the codon-based Z-test and the codon-based Fishers's exact test to compare the relative abundance of synonymous and non-synonymous

substitutions. The first test did not reject the null hypothesis of purifying selection, whereas that of neutral and positive selection was rejected; in Fisher's exact test, a p of 1.0 also supported purifying selection, rather than positive selection.

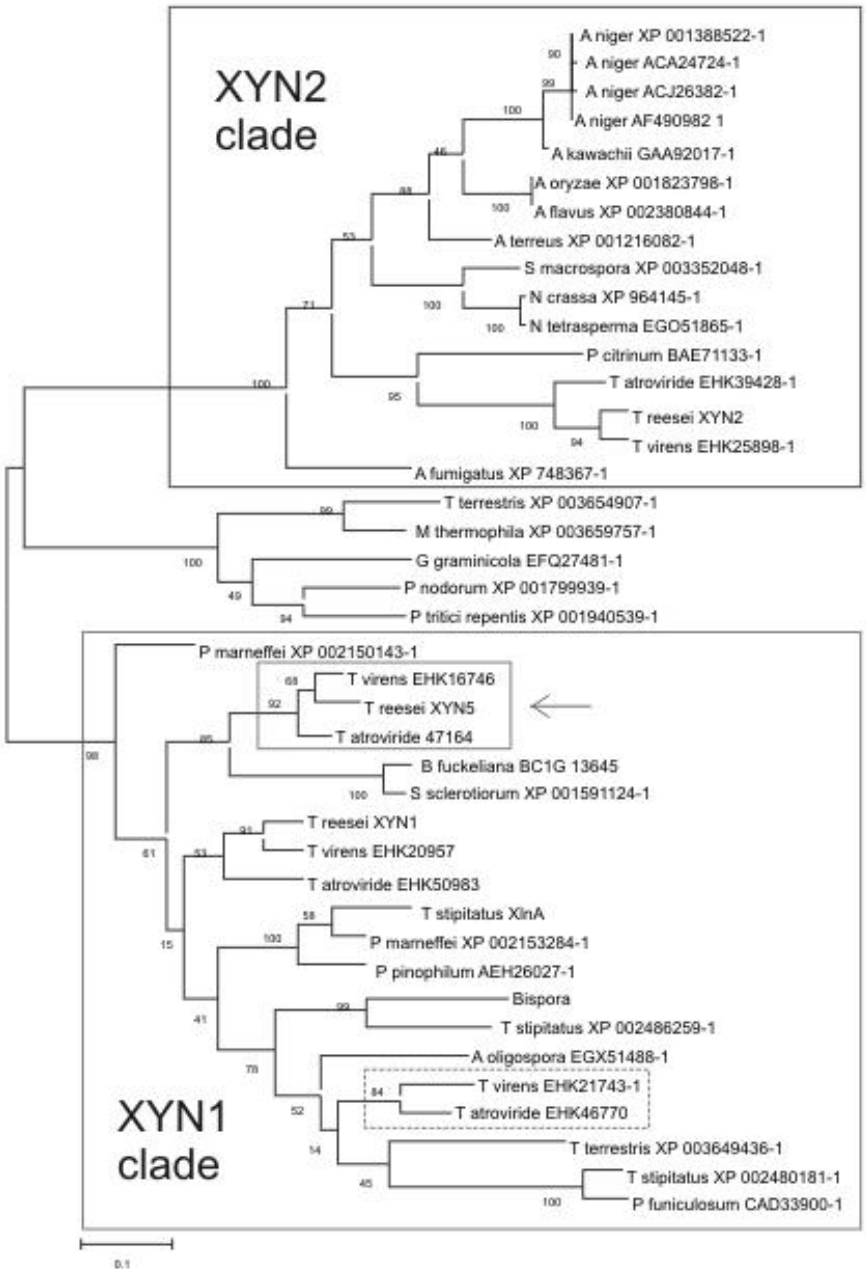

Fig. 3.1. Phylogenetic relationship of *T. reesei* xylanase XYN5 (GenBank accession number EGR44310) to other fungal xylanases of the GH11 family. A further clade consisting of additional GH11 xylanases lacking in *T. reesei* but present in *T. artoviride* and *T. virens* is marked by a dotted box. The XYN1 Numbers below nodes indicate the bootstrap value. The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions. GenBank Accession numbers of the respective proteins are indicated.

XYN1, XYN2, XYN4 and XYN5 are coregulated by D-xylose, XYR1 and CRE1

In order to identify the inducing conditions for the five *T. reesei* xylanases, we tested their potential induction by three different concentrations (0.5, 1 and 5 mM) of D-xylose in precultivated mycelia of *T. reesei* over a period of 6 hours. Consistent with earlier reports (9), D-xylose at 0.5 and 1 mM provided the highest xylanase transcript levels while 5 mM D-xylose already delayed xylanase induction. No induction was observed with xylitol which was due to the fact that it was not taken up readily under the present conditions (data not shown). For further induction experiments only 1 mM D-xylose was chosen, as this concentration also resulted in highest transcript levels in the different pentose pathway mutants.

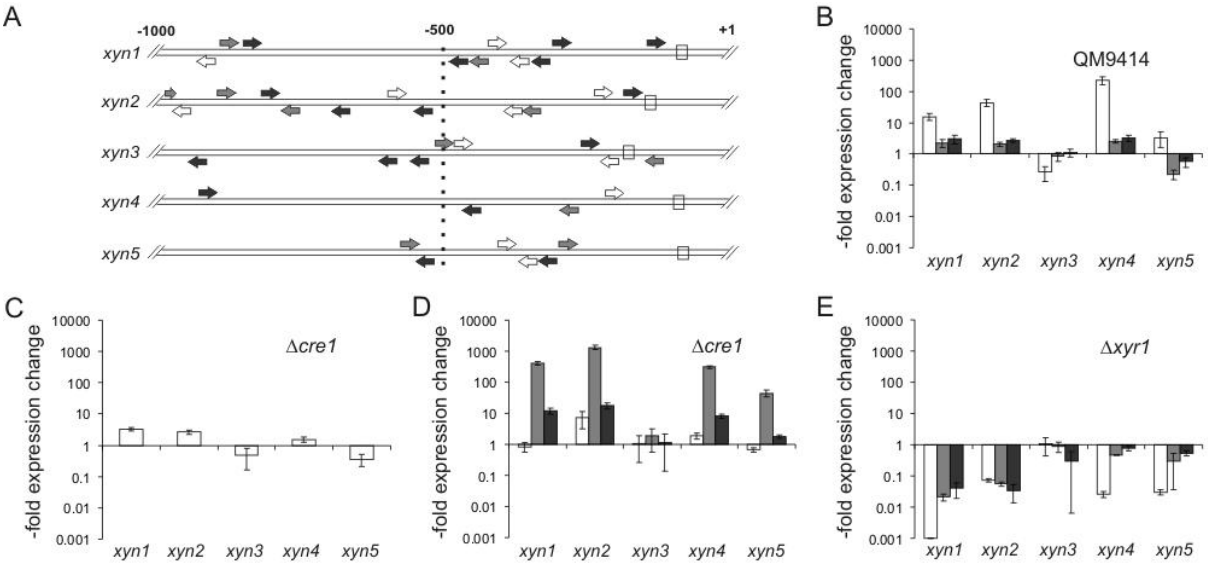

Fig. 3.2. Regulation of *T. reesei* xylanase gene expression. (A) Comparative analysis of regulatory motives present in the different xylanase promoter regions. Consensus sequences for XYR1 (white arrows; 5'-GGCTAA-3' or 5'-GGGTAA-3' as in the case of *xyn2*), HAP2/3/5 (grey arrows; 5'-CCAAT-3'), CRE1 (black arrows; 5'-SYGGRG-3') and the TATA box (white rectangle) are plotted on a region of 1000 bp upstream of the start codon. Arrows indicate the orientation of the respective motives present on either the sense strand or the antisense strand. (B-D) Relative expression levels of the five xylanases in *T. reesei* QM9414 and strains deleted in the carbon catabolite repressor CRE1 (Δ*cre1;* C-D) or the cellulase and xylanase regulator XYR1 (Δ*xyr1*; E). Precultivated mycelia were replaced to medium with 1 mM D-xylose (B, D, E; inducing conditions) or w/o carbon source (C). Samples were taken 2 h (white bars), 4 h (grey bars) and 6 h (black bars) after addition of the carbon sources. Relative expression values are plotted on a logarithmic scale where 1 indicates the transcription level of the reference strain QM9414 on medium w/o carbon source (B) or to the respective time point on inducing medium (C-E).

xyn1 and *xyn2* were previously reported to be subject to regulation by the C2H2 carbon catabolite repressor or CRE1 via a double lock mechanism (Mach *et al.* 1996; Mach-Aigner *et al.* 2008) that controls xylanase transcription at two different levels, by direct repression through CRE1, and indirectly through the CRE1-mediated repression of the transcriptional activator XYR1 (Marui *et al.* 2002).

Binding sites for the carbon catabolite repressor CRE1 are present in all xylanase genes (Fig. 3.2A), but only in *xyn1* they occur as an inverted repeat which has been proposed to be essential for carbon catabolite repression (Portnoy *et al.* 2011). We therefore tested the expression of all five xylanases in a Δ*cre1* strain: in the absence of an inducer *xyn1, xyn2* and *xyn4* were derepressed in the Δ*cre1* strain (Fig. 3.2C). Addition of the inducer D-xylose led to a further increase in transcript abundance for *xyn1, xyn2, xyn4* and *xyn5* (Fig. 3.2D) compared to the parent strain QM9414 indicating that for *xyn1, xyn2, xyn4* are subject to the double lock mechanism mentioned above but not *xyn5*.

The induction by D-xylose of all inducible xylanases was also coordinately dependent on the Zn2Cys6-type transcriptional activator XYR1, as the expression under D-xylose inducing conditions of all of them was undetectable in a Δ*xyr1* strain (Fig. 3.2E). This coordinated regulation of transcription was reflected by the presence of consensus binding motifs for XYR1 in all five xylanase upstream sequences (Fig. 3.2A). Also all contained binding sites for the HAP2/3/5 complex in vicinity of the XYR1 binding motif. A further binding site 5'-AGAA-3' was abundantly present in all five promoters. This site is bound by the putative XPP1 repressor (Würleitner *et al.* 2003) but its relevance for xylanase transcription, if any, is not clear at the moment and the data are therefore not included in Fig. 3.2A.

Impairment in D-xylose metabolism enhances induction by D-xylose

Having identified that D-xylose is able to coordinately induce four out of five xylanases in *T. reesei*, we wondered how a manipulation of the flux through the D-xylose catabolic pathway would influence uptake, intracellular polyol formation and their induction. As illustrated in Fig. 3.4A, this pathway involves the D-xylose /aldose reductase XYL1 (Seiboth *et al.* 2007a) and the xylitol dehydrogenase XDH1. The function of the latter can be partially replaced by the L-arabitol dehydrogenase LAD1 (Seiboth *et al.* 2003). LXR3 (Metz *et al.* 2013) connects D-xylose catabolism with L-arabinose catabolism via the reversible conversion of L-xylulose to xylitol, the first common intermediate.

We used isogenic knockout strains in the respective genes (*i.e.* Δ*xyl1,* Δ*lad1,* Δ*xdh1,* Δ*lad1/*Δ*xdh1,* Δ*lxr3*) and followed D-xylose uptake and intracellular sugar/polyol accumulation. As can be seen from some selected strains in Fig. 3.3 D-xylose was taken up at the highest rate in strain QM9414, while D-xylose could be detected in the medium of the Δ*lad1/*Δ*xdh1* strain still after 4 hours and in the Δ*xyl1* strain even after 6 hours. Uptake remained unaltered in the L-arabinose catabolic pathway mutant Δ*lxr3*. During the 6 hours of the experiment, xylitol – but no other polyol (such as L-arabitol) accumulated intracellularly (Fig. 3.3).

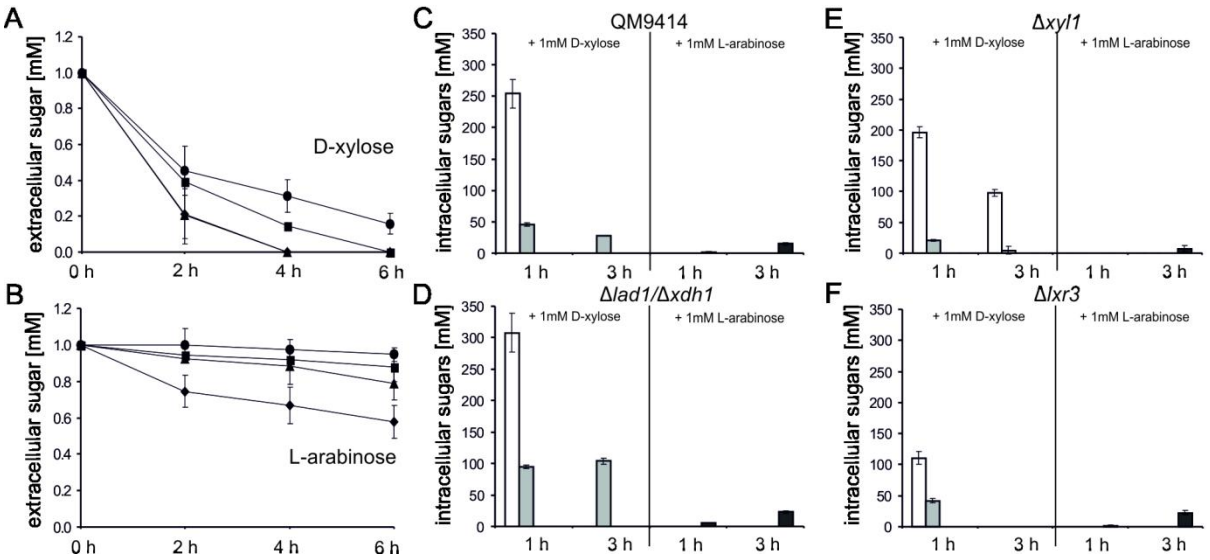

Fig. 3.3. D-xylose and L-arabinose uptake and intracellular accumulation of their corresponding polyols in *T. reesei*. D-xylose (A) and L-arabinose (B) concentration in the medium (QM9414, ♦; Δ*xyl1*, ●; Δ*lad1*/Δ*xdh1*, ■; Δ*lxr3* ▲). (C-E) Intracellular accumulation of D-xylose, xylitol, L-arabinose and L-arabinitol in *T. reesei* strains QM9414 (C) Δ*xyl1* (E), Δ*lad1*/Δ*xdh1* (D) and Δ*lxr3* (F). Strains were replaced to medium containing 1 mM D-xylose or L-arabinose.

As shown in Fig. 3.4B-E, the *T. reesei* strains bearing knockouts in *xyl1, xdh1, lad1* and *xdh1/lad1* all displayed significantly increased expression of *xyn1, xyn2, xyn4* and *xyn5*, and also – albeit less strongly - increased *xyn3* expression. In contrast, *T. reesei* Δ*lxr3,* which is deleted in the L-xylulose reductase LXR3 and therefore restricted in the conversion of xylitol to L-xylulose and further to L-arabitol, displayed xylanase expression levels comparable to that found in QM 9414 (Fig. 3.4F). These results indicate that the D-xylose reductase step is not essential for xylanase induction as reported earlier (Mach-Aigner *et al.* 2011). They also

show that the actual inducing component that mediates D-xylose induction of the five xylanases is a metabolite accumulating prior to the xylitol oxidation step as the double deletion strain Δ*lad1/*Δ*xdh1* completely blocks further catabolism of xylitol to D-xylulose. This strain is also not able to produce L-arabitol as the step from L-xylulose to L-arabitol is blocked. This conclusion is consistent with the fact that L-arabitol was not detected in the mycelia during these experiments indicating that L-arabitol cannot be the inducer formed from D-xylose under these conditions.

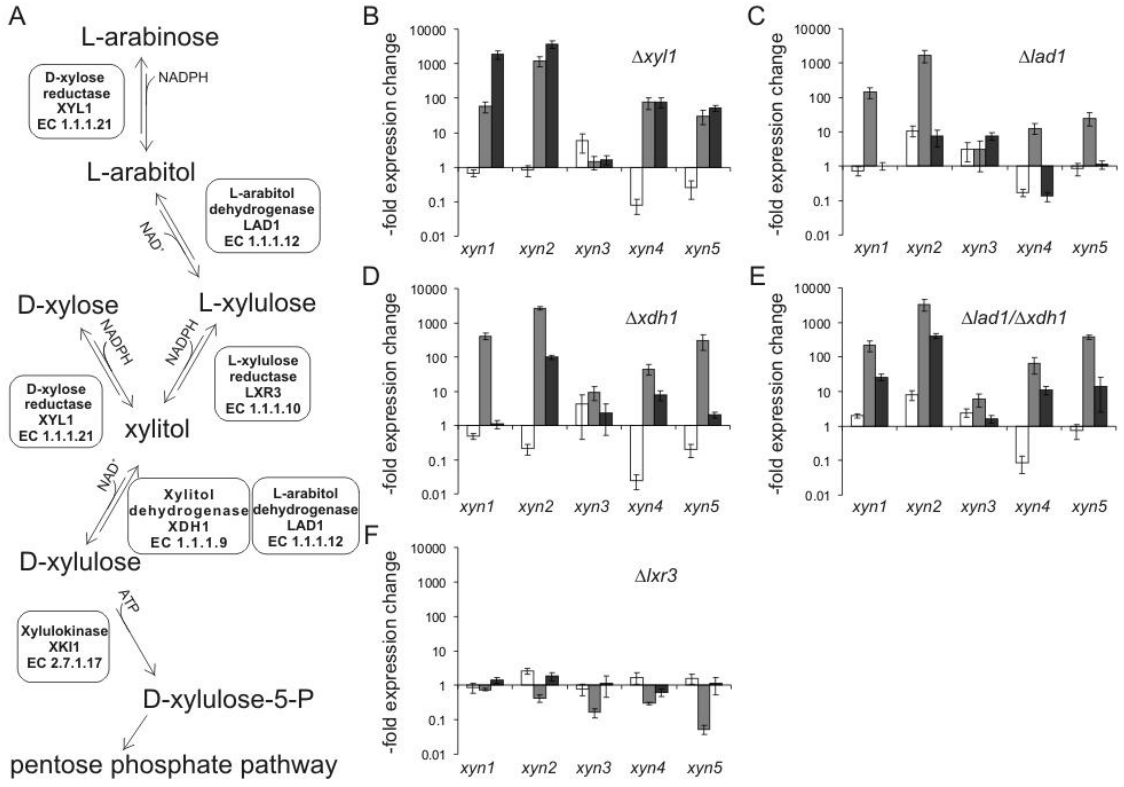

Fig. 3.4. Xylanase induction by D-xylose in strains deleted in specific steps of the D-xylose and L-arabinose pathway. (A) Scheme of the D-xylose and L-arabinose catabolic pathway in *T. reesei*. (B-F) Relative expression levels of the five xylanases (*xyn1* to *xyn5*) in *T. reesei* strains deleted in the D-xylose /aldose reductase (Δ*xyl1;* B), the L-arabitol dehydrogenase (Δ*lad1*; C), the xylitol dehydrogenase (Δ*xdh1*; D), in both dehydrogenases (Δ*lad1*/Δ*xdh1*; E) and the L-xylulose reductase (Δ*lxr3;* F). Strains were replaced to medium containing 1 mM D-xylose, and samples were taken 2 hours (white bars), 4 hours (grey bars) and 6 hours (black bars) after addition of the carbon source. Expression values, plotted on a logarithmic scale, are relative to strain QM9414 to the respective time point on inducing medium.

Xylanases are induced by L-arabinose and their transcription is enhanced in L-arabinose pathway mutants

Our data (*vide supra*) disapproved the hypothesis that L-arabitol is the true inducer for D-xylose mediated induction of xylanases in *T. reesei*. However, this does not exclude that xylanases could also be induced by the hemicellulosic pentose L-arabinose or a metabolite of its catabolism. Again, we used our set of isogenic knock-out strains to investigate how the carbon flux through these individual steps would influence induction. During these experiments, L-arabinose was taken up much slower than D-xylose, and only the polyol L-arabitol – but not xylitol - was found to accumulate in the mycelia (Fig. 3.3).

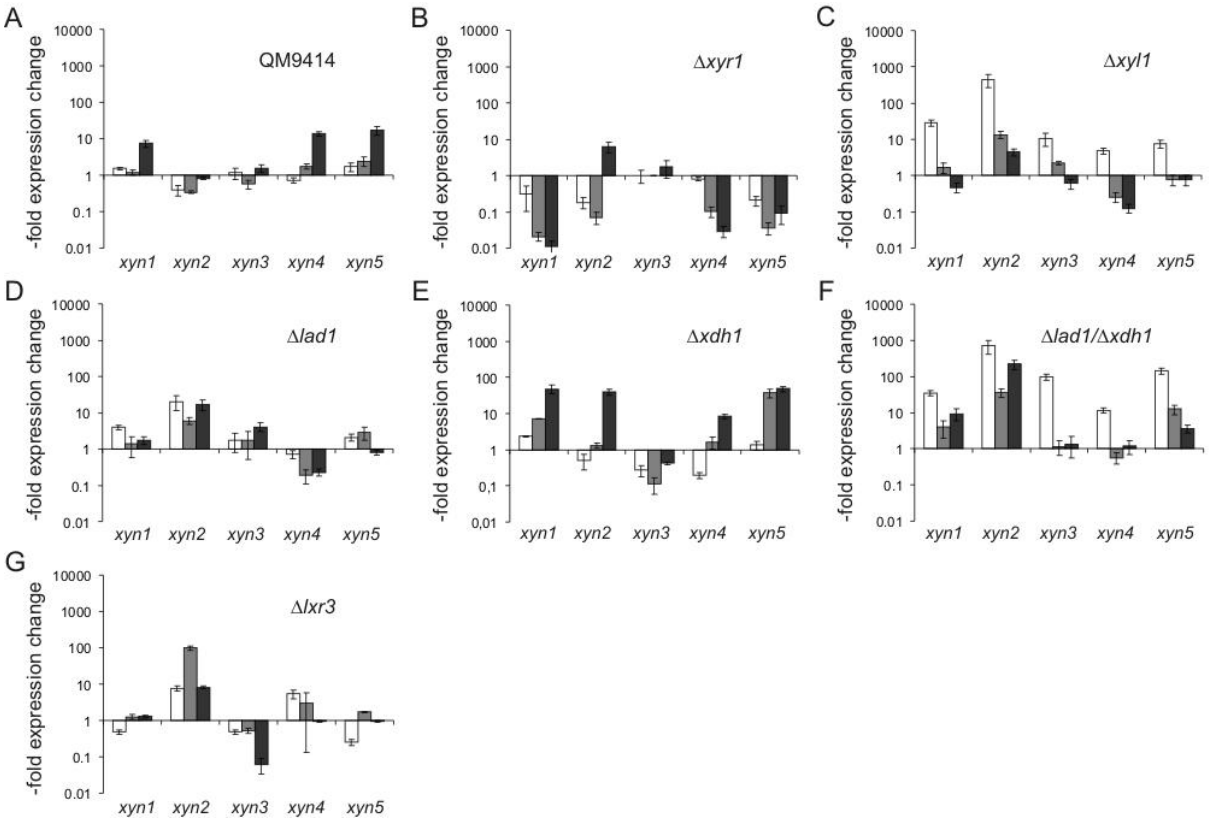

Fig. 3.5. Xylanase induction by L-arabinose: (A-F) Relative expression levels of the five xylanases *xyn1* to *xyn5* in the *T. reesei* QM9414 (A) and the deletion strains Δ*xyr1* (B), Δ*xyl1* (C), Δ*lad1* (D), Δ*xdh1* (E), Δ*lad1*/Δ*xdh1* (F) and Δ*lxr3* (G) replaced to medium containing 1 mM L-arabinose, plotted on a logarithmic y-scale. Samples were taken 2 hours (white bars), 4 hours (grey bars) and 6 hours (black bars) after addition of the carbon source. The values shown are related to the expression levels detected in QM9414 on medium without carbon source (A) or the transcriptional level of QM9414 to the respective time point on inducing medium (B-G).

Figure 3.5A and B show that the xylanases *xyn1*, *xyn4* and *xyn5* are indeed induced by L-arabinose and that this induction was also fully dependent on the general cellulase and hemicellulase regulator XYR1. Using our set of D-xylose/L-arabinose pathway mutants, we found that the induction by L-arabinose was again stimulated in the Δ*xyl1,* Δ*lad1,* and most strongly in the Δ*lad1/*Δ*xdh1* strains. In the Δ*lxr3* strain, the expression of only *xyn2* and *xyn4* was enhanced. Data obtained with the Δ*lad1* and Δ*lad1/*Δ*xdh1* strains indicate that induction by L-arabinose must be due to a metabolite that accumulates before the L-arabitol dehydrogenase step and that L-arabinose thus induces xylanase in a manner independent of that of D-xylose. In support of this, induction by L-arabinose could be further stimulated by addition of D-xylose and in the case of *xyn1* and *xyn2* the final induction was higher than the sum of individual inductions, demonstrating that their action is synergistic (Fig. 3.6).

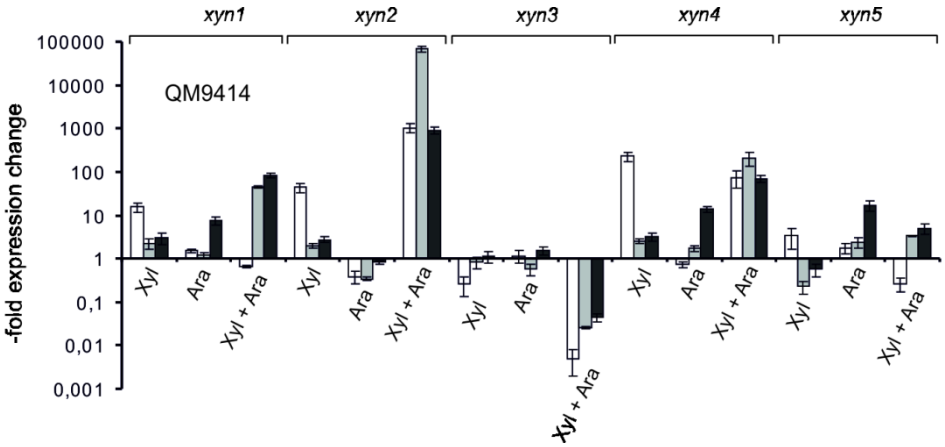

Fig. 3.6. Induction of xylanases by simultaneous presence of D-xylose and L-arabinose. Relative expression levels of the xylanases *xyn1 – xyn5* in the *T. reesei* reference strain QM 9414 replaced to medium containing 1 mM L-arabinose and 1 mM D-xylose. Samples were taken 2 hours (white bars), 4 hours (grey bars) and 6 hours (black bars) after addition of the carbon source. The values shown are related to the expression levels detected in the corresponding strains on 1 mM L-arabinose only.

Discussion

T. reesei contains three GH11 (XYN1, XYN2 and XYN5), one GH10 (XYN3) and at least one GH30 xylanase (XYN4). Based on the present results, all but XYN3 are coordinately induced by D-xylose or L-arabinose. XYN3 has previously been described to be only expressed in the QM 9414 mutant strain PC-3-7 on cellulose, L-sorbose and sophorose (Xu *et al.* 1998). While

our data confirmed the absence of D-xylose induced expression in the parent strain QM 9414, we nevertheless found notable expression upon induction by L-arabinose in strains blocked either in the reduction of L-arabinose *(Δxyl1*) or oxidation of L-arabitol (Δ*lad1*/Δ*xdh1*). We also observed induction by lactose in the parent strain QM 9414 (Ivanova *et al.* 2013). Hence this is the only one out of the five xylanases that escaped regulation by D-xylose and L-arabinose. The reason for this is unknown, as the enzymatic properties of XYN3 – which may shed a light on its natural substrate – have not been investigated as yet. We should also like to note that our present data were obtained with the low cellulase producer QM 9414, which is a two-step mutant from QM 6a. Although the differences in the genome sequence between QM 9414 and QM 6a are not present in genes that could be hypothesized to play a role in xylanase regulation (Vitikainen *et al.* 2010), we cannot rule out that the regulation in wild-type strains of *T. reesei* could differ in minor aspects.

Another interesting finding that emerged from our studies is that the inducing concentration of D-xylose appears to be by far more critical for induction in comparison to other fungi such as *A. niger* (Mach-Aigner *et al.* 2012) and *N. crassa* (Sun *et al.* 2012) as D-xylose lead to catabolite repression of xylanase formation already at comparatively low concentrations.

Our data provide a new model for xylanase regulation with respect to the potential inducer(s) and the contribution of the enzymes of the catabolic pathways for D-xylose and L-arabinose in inducer formation. We clearly show that D-xylose and L-arabinose induce xylanases via different compounds: while some enzymes of the catabolic pathway for L-arabinose and D-xylose can act in both pathways (e.g. XYL1, XDH1), LXR3 is the specific step that connects both pathways. Thus the phenotype of the Δl*xr3* strain allows to distinguish between the contributions of the two pathways, as it blocks the interconversion of the two sugars. Consequently, the observed induction by D-xylose and L-arabinose in the Δ*lxr3* strain must be due to different inducing metabolites. Further support for this conclusion comes from the double deletion strain Δ*lad1/*Δ*xdh1* which is completely blocked in the xylitol to D-xylulose step and the L-arabitol to L-xylulose step. This strain shows a strong stimulation of xylanase transcription for both pentoses.

The strong inducing effect observed by D-xylose and L-arabinose in a Δ*xyl1* strain where the major enzyme for D-xylose and L-arabinose reductase activity is missing would support the conclusion that both D-xylose and L-arabinose are the “true inducers” since both pentoses stimulate induction without being efficiently metabolized. Such an interpretation would be

in accordance with data from *N. crassa* where a deletion of the *xyl1* orthologue impairs growth on D-xylose but stimulates expression of an endoxylanase (Sun *et al.* 2012). The equally strong stimulation of induction by D-xylose and L-arabinose in the Δ*lad1/*Δ*xdh1* strains, however, makes the identification of the true inducer more difficult and would argue in favor of xylitol or L-arabitol. It is therefore possible that the protein interacting with the inducer can bind both components. Akel *et al*. (Akel *et al.* 2009) have recently demonstrated that the expression of α-L-arabinofuranosidase genes in *T. reesei* requires a cross talk between L-arabinose or L-arabitol and the aldose reductase XYL1, and the present data are also compatible with the operation of such a mechanism.

The above data (*i.e.* that the absence of xylose reductase or xylitol/L-arabitol dehydrogenases leads to increased xylanase gene expression) contradicts recent data by Mach-Aigner *et al.* (Mach-Aigner *et al.* 2010; Mach-Aigner *et al.* 2011). One argument to explain these differences is that they used only a single time point for transcript analysis, at which expression in the Δ*xyl1* and Δ*xdh1/*Δ*lad1* does not yet take place and was therefore overlooked. However, their claim that L-arabitol is the inducer of xylanases in the presence of D-xylose can clearly not be maintained: L-arabitol did not accumulate in any of the mutant strains when incubated on D-xylose, and this appears logical because strains nonfunctional in LXR3 and LAD1/XDH1 cannot metabolize D-xylose to L-arabitol.

In this paper, we have also shown that L-arabinose can act as an inducer of xylanase gene expression and that this induction is also dependent on XYR1. While this finding is new, it was previously shown that a blockage of the L-arabinose pathway results in the upregulation of L-arabinose inducible α-L-arabinofuranosidases or β-xylosidases in *A. nidulans* or *T. reesei* (de Vries *et al.* 1994; Akel *et al.* 2009). In *T. reesei* L-arabinose induced expression of *abf2* and *bxl1* is dependent on XYR1 while the expression of two *abf1* and *abf3* was only slightly affected. In Aspergilli a second transcriptional regulator AraR responds to the presence of L-arabinose and activates L-arabinose releasing enzyme transcription (Battaglia et al., 2011 Applied Microb and Biotech). AraR is, however, specific for the *Eurotiales* and no orthologues are present in *T. reesei*. In *T.reesei* the inducing effects by L-arabinose on xylanase gene expression are part of the XYR1 circuit and future research in *Aspergilli* will show if xylanases are induced by L-arabinose and if this regulation is via XlnR or AraR. Our data also show that this induction is not simply due to the formation of the same inducing metabolite from D-xylose and L-arabinose as suggested earlier (Mach-Aigner *et al.* 2011), as

further supported by the additive effect of induction by D-xylose and L-arabinose. The advantage of having two inducers may come from the fact that there are two major types of xylans: one, which occurs in the cell wall of cereals, which often contain large quantities of L-arabinose and are consequently termed arabinoxylans. In contrast, hardwood xylans contain large amount of D-glucuronic acid linked to the backbone and are named glucuronoxylans (Scheller and Ulvskov 2010). *T. reesei* appears to be more adapted to the latter because of its amplification of GH67 α-methyl-glucuronidases and GH30 glucuronyl-xylanases in its genome (Kubicek 2012b). The availability of an arabinoxylan may therefore be signaled by L-arabinose removed from the arabinoxylan side chains, whose removal will make the xylan backbone available for hydrolysis.

Chapter 4

Metabolic Engineering of Inducer Formation for Cellulase and Hemicellulase Gene Expression in Trichoderma reesei.

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Metabolic engineering of inducer formation for cellulase and hemicellulase gene expression in *Trichoderma reesei*.

Seiboth B, Herold S, Kubicek CP

Contribution of the author:

SH made the figures, wrote the chapter *Engineering the Pentose catabolic pathways for D-xylose and L-arabinose, critical read and* revised the whole manuscript

The filamentous fungus *T. reesei* is today a paradigm for the commercial scale production of different plant cell wall degrading enzymes mainly cellulases and hemicellulases. Its enzymes have a long history of safe use in industry and well established applications are found within the pulp, paper, food, feed or textile processing industries. However, when these enzymes are to be used for the saccharification of cellulosic plant biomass to simple sugars which can be further converted to biofuels or other biorefinery products, and thus compete with chemicals produced from fossil sources, additional efforts are needed to reduce costs and maximize yield and efficiency of the produced enzyme mixtures. One approach to this end is the use of genetic engineering to manipulate the biochemical and regulatory pathways that operate during enzyme production and control enzyme yield. This review aims at a description of the state of art in this area.

Introduction

Fungi are for a long time exploited for the industrial production of a diverse range of metabolites and enzymes. The decomposition of cellulosic plant biomass by fungi is only one example which demonstrates the importance of this group of organisms for our life on earth by the cycling of carbon and it shows how such essential processes can be exploited for industrial applications to produce biofuels and other biorefinery products from renewable carbon sources. Biorefining the sustainable processing of biomass into a spectrum of bio-based products and bioenergy may well play a major role in producing chemicals and materials which are today still produced from oil. Active research efforts in different areas will be necessary to make these processes cost-effective. One of the key issues for biotechnologists is the identification of novel enzymes, improvement of physicochemical properties and the efficient production of these enzymes in appropriate host organisms. A key to the latter is to understand under which conditions the host produces these enzymes to design strains with improved enzyme production properties. Therefore fundamental research is necessary to identify regulatory and metabolic networks which control the expression of the extracellular enzymes with the ultimate goal to improve the enzyme yield.
Fungi secrete a wide variety of extracellular enzymes to degrade different plant polymers including cellulose, hemicellulose, pectin and lignin which can be used by the fungus as carbon and energy source. Most of these enzymes active on the polysaccharides are known

today under the name CAZymes (carbohydrate active enzymes) and the CAZy database is dedicated to collect genomic, structural and biochemical information (Cantarel *et al.* 2009). Beside their role as an attractive resource for new enzymes, different fungi such as *Aspergillus* spp. or *Trichoderma reesei* are also used routinely as an industrial production host for these enzymes since they are well adapted to fermenter cultivations and secrete vast amounts of enzymes which are not reached by other microorganisms.

The filamentous fungus *T. reesei* is today a paradigm for the commercial scale production of different plant cell wall degrading enzymes mainly cellulases and hemicellulases. Maybe the most important and therefore best studied cellulase today is CEL7A/CBH1 of *T. reesei* for which also the first 3D model became available (Divne *et al.* 1994). The fungus and its enzymes have a long history of safe use in industry and well established applications are found within the pulp, paper, food, feed or textile processing industries. Today its enzymes are also employed for the saccharification of cellulosic plant biomass to simple sugars which can be further converted to biofuels or other biorefinery products (Harman and Kubicek 1998; Bouws *et al.* 2008; Kumar *et al.* 2008a). Although *T. reesei* is already the main industrial source for such enzymes (Merino and Cherry 2007) and despite the fact that industrial strains produce more than 100 g/L of cellulases (Cherry and Fidantsef 2003), additional efforts are needed to reduce costs and maximize yield and efficiency of the produced enzyme mixtures (Carroll and Somerville 2009; Wilson 2009). Beside this prominent role in industrial applications, *T. reesei* serves today as a model organism for the regulation and biochemistry of (hemi)cellulose degradation (Aro *et al.* 2005; Stricker *et al.* 2008b; Kubicek *et al.* 2009). Surprisingly, the genome sequence of *T. reesei* QM6a revealed that the fungus possess only a rather small set of cellulases and hemicellulases in comparison to other plant biomass degrading fungi. This underscores our still poor understanding of the process of cellulose degradation (Martinez *et al.* 2008).

Initiating the degradation of cellulose

One of the major obstacles in the degradation of plant cell walls is that their components are long polymers and often insoluble which prevents a direct uptake into the cell. Therefore *T. reesei* and other celluloytic fungi have developed an arsenal of extracellular enzymes – including cellulases and hemicellulases – which specifically degrade the different

polysaccharides. But the production of extracellular enzymes is a highly energy consuming process and includes beside their transcription and translation – processes necessary for all proteins - also complex posttranslational modification steps such as *N* or *O*-linked glycosylation which are accomplished during the passage of these enzymes through the secretory pathway. These processing steps are completed by the export of the active enzymes from the fungal cell into the environment to cleave the plant biomass polymers. Therefore the expression of genes encoding such CAZymes is a tightly regulated process to minimize the energy loss for the fungus. To survive in such an ecosystem *T. reesei* has developed sophisticated mechanisms to guarantee an economic production of these enzymes.

The degradation of different carbon sources that are present as complex mixtures follows an energy driven hierarchy which implies the presence of an efficient sensing and signaling system to detect the type (or composition) of the plant biomass. The regulation of enzyme expression is thus controlled by two major control circuits, i.e. specific induction and general carbon catabolite repression (CCR). Since most of the investigations on CCR have been performed with D-glucose, this type of repression is often termed glucose repression. However, this would be an oversimplification since also other carbon sources are able to provoke CCR, and in fact the rate of uptake is probably more critical for it than the sugar itself (Portnoy *et al.* 2011). As a result, most of the CAZyme genes necessary for the fragmentation of the plant cell wall polymers are repressed by the presence of increased concentrations of fast metabolizable carbon sources that can arise from the degradation of these polymers including D-glucose, D-galactose, D-xylose or others. This mechanism ensures that the extracellular rate of hydrolysis of the polymers does not occur in access to uptake and intracellular metabolism of the break-down products, and ensures that other microorganisms present in this habitat do not benefit from the degradation efforts of *T. reesei* by competing for the different break-down products.

Most of the enzymes for plant cell wall degradation including cellulases and hemicellulases are usually only formed adaptively in the presence of their substrates. Therefore effective sensing and signaling networks must be present to communicate the presence of the substrate to the fungus. As a consequence, induction of the different degradative enzymes is initiated. Since this process is essential for the survival of the fungus in its natural habitat, it is likely that it has developed several strategies that ensure the sensing of the lignocellulosic

substrates in its environment. One of these strategies is the formation of minute amounts of CAZymes constitutively. These enzymes are able to initiate degradation of cellulose, thereby releasing small amounts of oligosaccharides which are then taken up by the fungus to induce further cellulase biosynthesis. Evidence for this theory comes from the finding that addition of anti-cellulase antibodies block induction of cellulase formation by cellulose (El-Gogary *et al.* 1989) and that low levels of the major cellobiohydrolases *cel7A* and *cel7B* are present under non-inducing conditions (Carle-Urioste *et al.* 1997). A microarray analysis identified further candidate genes including *cel5b* that displayed a similar low basal expression level in the absence of cellulose (Foreman *et al.* 2003). This cellulase contains a putative binding site for the attachment of a glycosylphosphatidylinositol anchor which would allow a location of the enzyme close to the cell membrane and make it therefore an interesting candidate for inducer formation. A second strategy to initiate cellulose degradation is connected to the production of conidia, the asexual form of spores in fungi. On the surface of these conidia an array of different plant polymer degrading enzymes is found including cellulases and hemicellulases (Kubicek 1987; Messner *et al.* 1991). Their removal by nonionic detergents impairs germination of the conidia on cellulose (Kubicek *et al.* 1988) and it was also been shown that a *T. reesei* strain lacking the two main cellobiohydrolases is unable to initiate germination in the presence of cellulose (Seiboth *et al.* 1997).

Metz et al. (2011) showed that numerous CAZyme genes are transcribed during the early phase of conidiation in the absence of any exogenous inducer and that their formation depends on the major cellulase and hemicellulase transcriptional regulator XYR1 (see below). Absence of XYR1 impairs germination of the spores on cellulose. The strong presence of different CAZymes might be a

peculiarity of *T. reesei* since during conidiation of other fungi, *i.e. Neurospora crassa* or *Aspergillus fumigatus* no upregulation of cellulases or hemicellulase genes was detected. This strong presence of the different CAZymes on the conidia provides a selective advantage for *T. reesei* in the colonization of its lignocellulose rich habitat. A third possibility to sense cellulose is suggested by the finding that cellulose transcripts are present in *T. reesei* mycelia 20–30 h after complete consumption of the carbon source (Ilmen *et al.* 1997). This phenomenon is not simply due to a relief from carbon catabolite repression and is not found after transfer to media lacking any carbon source. A conclusive interpretation for this observation is still missing, but it is possible that during this time carbohydrates (e.g. b-

glucans) are released from the fungal cell wall which may induce cellulase formation. Alternatively, it is possible that this is due to the initiation of chlamydospore formation: *T. reesei* does this after exhaustion of a fast metabolizable carbon source, and it is thus possible that the detected cellulase transcripts are formed during chlamydospore development analogously to their appearance during conidiation (Metz *et al.* 2011). Although different break-down products of the polymers might be produced by the action of other microorganisms or by abiotic factors in *T. reesei*'s natural environment, these three scenarios underscore how effectively *T. reesei* is adapted to sense cellulose and related polymers in its environment. As a consequence of all these models for initiation of cellulose degradation different cellulose break-down products are produced which can serve as a specific inducer to boost the expression of further cellulases. The products of the synergistic action of the different cellulases are glucooligosaccharides, cellobiose and (in the case of high b-glucosidase activities) D-glucose. It would be logic to assume that one of these or a further metabolite thereof is the actual inducer of cellulases. D-glucose can be ruled out for a number of reasons. Beside that it causes strong carbon catabolite repression, it is a carbohydrate that can be also derived from other polysaccharides (such as starch) as well and is therefore not a specific signal for the presence of cellulose. This is in accordance with findings that even a low feeding of D-glucose does not induce cellulose induction (Karaffa *et al.* 2006). On the other hand, growth in the presence of cellobiose has indeed been shown to induce cellulose expression in fungi including *T. reesei* but did not so in other fungi (Aro *et al.* 2005). A reason for the absence of induction in some cases could be the activity of extracellular b-glucosidase, which cleaves: cellobiose to D-glucose, which inhibits cellobiose transport (Kubicek *et al.* 1993) and also represses cellulase expression via CCR.

Inhibition of transport may be the more relevant mechanism because it has been shown that altering the relative ratio of the uptake and hydrolysis rate (*e.g*. by the addition of β-glucosidase inhibitors, lowering of the pH below 3 which reduces β-glucosidase activity, or the use of β-glucosidase knock-out strains) increases cellulose induction by cellobiose (Sternberg and Mandels 1979; Fritscher *et al.* 1990; Fowler and Brown 1992). Since growth of the fungus on cellulose releases cellobiose in concentrations that favor the uptake rather than hydrolysis (the K_m of β-glucosidase for cellobiose is 20–40-fold higher than that of the b-glucoside permease; Kubicek *et al*. 1993), cellobiose could well be the in vivo inducer. The most well studied inducer of cellulase formation in *T. reesei* is, however, sophorose (α-

glucosyl-β-1,2-D-glucoside). It is the strongest known soluble inducer of cellulases for *T. reesei*, and its presence in the culture medium during growth on cellulose has been demonstrated (Mandels *et al.* 1962) as well as its formation by β-glucosidase via transglycosylation (Vaheri *et al.* 1979).

Absence of the major extracellular b-glucosidase CEL3A resulted in a delay in induction of the other cellulase genes by cellulose, but not by sophorose, and a *cel3a*-multicopy strain formed higher amounts of cellulases than the parent strain under non-saturating concentrations of sophorose (Kubicek *et al.* 2009). However, also other β-glucosidases including the intracellular CEL1A are able to produce sophorose and cellobiose from glucose, and could therefore be involved in inducer formation. A potentially important property of sophorose is that it is much less efficiently hydrolysed by b-glucosidases, while still efficiently transported by the *T. reesei* cellobiose permease. Thus sophorose could be a gratuity inducer that mimicks the role of cellobiose in nature.

Engineering the regulatory systems of cellulase and hemicellulase expression

Specific transcriptional activation and repression

One key to improve the production of specific enzymes or a group of enzymes is to modulate the regulation of these enzymes on the level of transcription by overexpressing transcriptional activators or deactivating transcriptional repressors. A key to this is to understand the different regulatory networks which signal the presence, composition and abundance of possible substrate to the fungal nucleus to initiate or terminate the expression of the extracellular enzymes for polymer degradation, the sugar transporters and the intracellular metabolic enzymes for carbon assimilation or energy production. The obligatory presence of an inducer for high cellulase gene expression implies tight regulation of their promoters. Most of the cellulase genes are also regulated in a coordinated way, although the relative ratio of their expression may differ in higher production mutants (Foreman *et al.* 2003). At least three transcriptional activators including XYR1, ACE2 and the HAP2/3/5 complex, as well as the two repressors CRE1 and ACE1 are involved in cellulase regulation.

XYR1 is clearly the major player in n the transcriptional regulation of cellulolytic and xylanolytic enzymes. Absence of this zinc binuclear cluster transcriptional activator virtually eliminates their expression by all known inducers (Stricker *et al.* 2006). XYR1 regulates also

some α-L-arabinofuranosidases (Akel *et al.* 2009) and the catabolism of D-xylose, L-arabinose, lactose via induction of the reductase XYL1 and catabolism via XYL1 and the β-galactosidase BGA1 (Seiboth *et al.* 2007a; Stricker *et al.* 2008b). Regulation is accomplished by binding to its target promoters by a GGC(T/A)4 consensus (Furukawa *et al.* 2009). Although necessary for both cellulase and xylanase induction it is not clear how XYR1 is able to selectively induce cellulases or xylanases. As a transcriptional activator XYR1 must receive respective signals from the different inducers but the exact mode of activation is not known today. Noguchi *et al.* showed that in the absence of D-xylose the *Aspergillus niger* XYR1 homologue XlnR was present as a mixture of different phosphorylated forms while addition of D-xylose caused additional phosphorylation of the protein (Noguchi *et al.* 2011). It is however interesting that in the hypercellulolytic strain CL847 an upregulation of *xyr1* transcription during growth on lactose was observed compared to other improved cellulase producers (Portnoy *et al.* 2011). Therefore the genetic background in which *xyr1* is expressed might play a role for the improvement of (hemi)cellulase production.

Beside XYR1 the zinc binuclear cluster type transcriptional activator ACE2 regulates (hemi)cellulase expression. Its deletion lowers the induction kinetics for different cellulase transcripts and reduces the cellulase activity to 30–70% when grown on cellulose. However, it does not affect cellulase induction by sophorose (Aro *et al.* 2001). ACE2 binds in vitro to the 5'-GGCTAATAA-3' site present in the *cel7A* promoter. Therefore, both XYR1 and ACE2 are able to bind the same motif. Using the xylanase *xyn2* as a model system, Stricker *et al*. (2008b) suggested that ACE2 acts in a dual role by (i) antagonizing early induction and (ii) enhancing of a continuous extension of *xyn2* expression. ACE2 *xyn2* promoter binding is dependent on its phosphorylation and dimerization. It is possible that ACE2 has evolved as a fine tuning component that binds to XYR1 and enables its response to the different inducing signals, as its gene knock out did not significantly affect cellulase gene transcription. Induction of the *T. reesei* cellulase gene *cel6A* by sophorose is partially dependent on a CCAAT box located adjacently to its XYR1/ACE2 binding site. This motif is bound by the ubiquitously occurring trimeric HAP2/3/5 complex (Zeilinger *et al.* 2001) which contains a histone fold motif, suggesting an involvement in chromatin mediated regulation, eventually by association with acetyltransferases. No efforts to modify their expression were reported. In contrast to the regulation of (hemi)cellulases in other fungi additional specific transcriptional repressors for cellulase or hemicellulase genes have been described for *T.*

reesei, *i.e*. ACE1 and XPP1. ACE1 a Cys2His2-type zinc finger repressor and binds to the core sequence 5'-AGGCA-3' (Saloheimo *et al.* 2000). Deletion of *ace1* increased the expression of all the main cellulase and xylanase genes in sophorose and cellulose induced cultures (Aro *et al.* 2003). A possible explanation for its action could be that the main regulator *xyr1* was found to be upregulated on D-xylose in a *ace1* deleted strain (Mach-Aigner *et al.* 2008). A strain deleted in both *ace1* and *ace2* expressed cellulases and xylanases similar to the *ace1* deleted train (Aro *et al.* 2003). The importance of ACE1 in cellulase production is also emphasized by the finding that the hyperproducing mutant *T. reesei* CL847 exhibits beside the above mentioned *xyr1* upregulation a strongly reduced *ace1* expression (Portnoy *et al.* 2011). A hint to a possible function of ACE1 comes from work with its orthologue in *Aspergillus nidulans (Chilton et al. 2008).* Its orthologue StzA was described here as a stress response factor and evidence of competition, or interaction, between StzA and AreA binding sites in promoters of *stzA*, as well as genes involved in the metabolism of amino acids was provided. Potential StzA binding sites were identified in *cpcA* (cross pathway control regulator of amino acid biosynthesis) and the presence of potential CpcA binding sites (5'-TGAC-3') in the *stzA/ace1* promoters suggests an intriguing link between intracellular amino acid availability and cellulase gene expression. Support for a cross-talk between amino acid availability and cellulase formation comes from (Gremel *et al.* 2008) which reported an enhancement of cellulase gene expression by the addition of methionine in *T. reesei*. Analysis of the *xyn2* promoter suggested the binding of another putative repressor protein XPP1 (Mach-Aigner *et al.* 2010) to an AGAA-box under glucose repressing conditions. A *xpp1* gene knock out is still required to see the potential of this putative regulator in xylanase gene transcription.

Wide domain regulators

Different wide domain regulators govern the expression of the different cellulases and hemicellulases. Their corresponding genes are usually not expressed during growth on glucose even in the presence of an inducer. This has been shown to be due to both inducer exclusion (the inhibition of inducer uptake by D-glucose) and glucose repression, the latter generally called carbon catabolite repression (CCR). Consequently, one of the earliest attempts to improve cellulase production was the removal of CCR. Classical mutagenesis combined with selection for 2-desoxyglucose resistance under inducing conditions has led to

increased cellulase producers such as *T. reesei* RUT C30, RL-P37 and CL847, thus supporting the importance of CCR in cellulase formation. On the transcriptional level the key players for CCR is the Cys2His2 type transcription factor CRE1 (Ilmen *et al.* 1996). Numerous cellulase, hemicellulase and pectinase genes have been shown to be regulated by CRE1 in *T. reesei* (Aro *et al.* 2005). It is therefore not surprising that the analysis of RUT-C30 including its derivative CL847 identified a loss of a 2478-base pair fragment, which starts downstream of the region encoding the CRE1 zinc finger (Ilmen *et al.* 1996; Seidl *et al.* 2008). In general, mutations of the *cre1* gene or of its binding motif 5'-SYGGRG lead to a (partial) derepression of enzyme gene expression on glucose as demonstrated for *cbh1* and *xyn1* (Aro *et al.* 2005; Kubicek *et al.* 2009). Functional CRE1 binding sites have been shown to consist of two closely spaced 5¢-SYGGRG motifs, and it has been suggested that direct CRE1 repression would occur only through such double binding sites. Phosphorylation of a serine residue in a conserved short stretch of *T. reesei* CRE1 has been demonstrated to regulate its binding to its target sequence (Cziferszky *et al.* 2002).

Relieve from carbon catabolite repression alone is not sufficient for high cellulase production and cultivation on d-glucose or other carbon sources results only in low cellulase levels, indicating that cellulase hyperproduction is still inducer dependent. About sixfold-higher cellulase and 2.5-fold-higher xylanase levels were found during bioreactor cultivations (Nakari-Setälä *et al.* 2009). A further gene *cre2* is involved in cellulase gene repression and *cre2* deletion strains are consequently derepressed (Denton and Kelly 2011). CRE2 is orthologous to the *A. nidulans* CreB and involved in deubiquitinylation. These cysteine proteases target proteins for degradation by the proteasome. CreB forms a complex with the WD40-repeat protein CreC to protect CreB against proteolysis in the absence of carbon catabolite repression. One of the highlights from the analysis of the genome sequence of the *T. reesei* genome (Martinez *et al.* 2008) was that – in contrast to all other ascomycetes whose genome had been sequenced – its cellulase, hemicellulase and other CAZyme genes were to be located in several discrete cluster. The reason for this and its potential advantage remained unclear.

However, it was conspicuous that a number of genes for secondary metabolite production such as non-ribosomal polypeptide synthases and polyketide synthases were found in the same clusters. Such secondary metabolite cluster genes often occur near the telomere end of the chromosomes and have been demonstrated to be regulated at the level of chromatin

remodelling by a the putative protein methyltransferase LaeA in *A. nidulans* (Reyes-Dominguez *et al.* 2010). Because of the co-clustering of CAZymes with secondary metabolite synthesis genes in the *T. reesei* genome, we hypothesized that cellulase expression may be regulated by a *T. reesei* LaeA orthologue. Production of cellulases and other CAZymes was indeed reduced almost to zero in the absence of LAE1, and dramatically increased by its overexpression showing that LAE1 is essential for cellulase and hemicellulase formation in *T. reesei* (Seiboth *et al.* 2012b). The role of LAE1 in cellulose regulation is, however, less clear and deserves further efforts.

A summary of the events involved in the regulation of cellulase and hemicellulase induction by cellulose if given in Fig. 4.1.

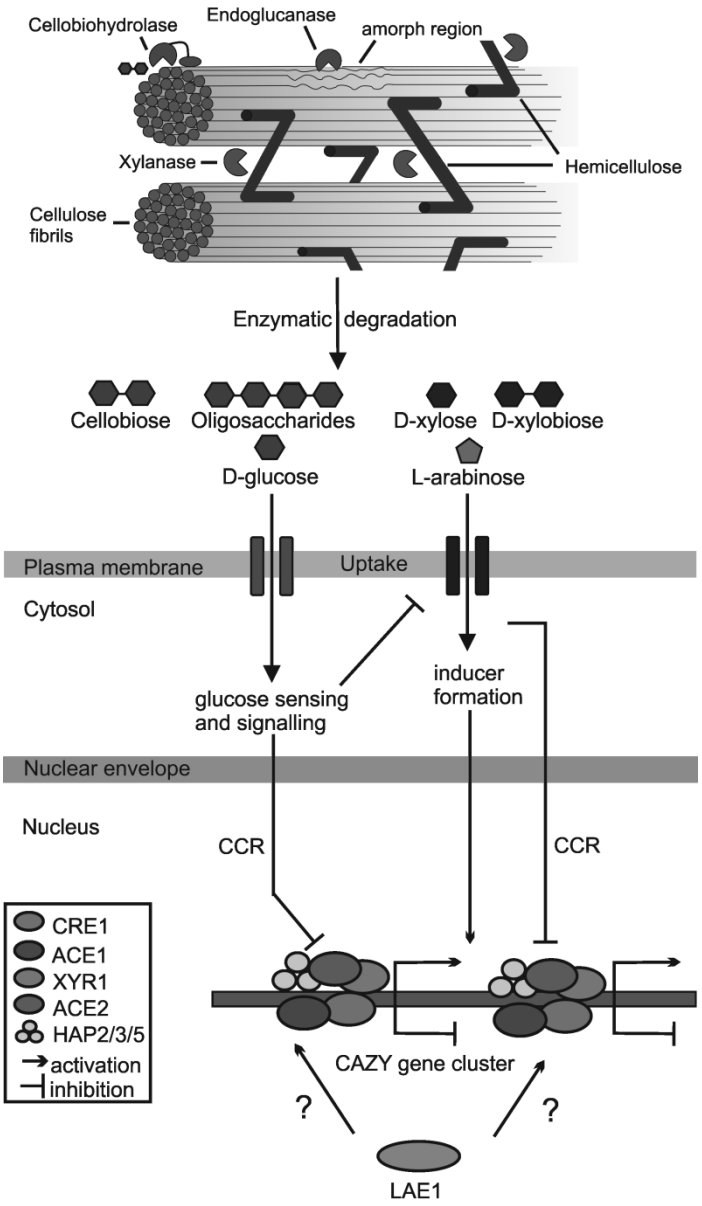

Fig. 4.1 Summary of steps involved in the regulation of cellulase and hemicellulase gene expression in *T. reesei.*

Engineering the signal pathways for cellulase and hemicellulase gene expression

Although the regulatory network that governs (hemi)cellulase expression on the transcriptional level is already understood in some detail, the order of events by which different inducers and repressors signal their presence to modulate the different transcriptional regulators (e.g. by phosphorylation) is hardly known. The exogenous addition of dibutyryl-cyclic AMP or of inhibitors of cAMP-phosphodiesterase, to enhance intracellular

cAMP levels, lead to secretion of increased endoglucanase activities in *T. reesei* (Sestak and Farkas 1993). cAMP, a classical messenger, is formed by the action of adenylate cyclase which in turn is activated by the G-alpha subunit of the G-protein complex associated with the cytoplasmic C-terminus of transmembrane receptors that receive the external signal to be transmitted. In *T. reesei*, the G-alpha proteins GNA1 and GNA3 seem to be only partially involved in cellulose gene expression: while loss of function strains and strains bearing constitutively activated *gna1* and *gna3* alleles showed a decreased and increased *cel7A* and *cel6A* transcription under some conditions, respectively, cellulase gene expression was still dependent on the presence of an inducer, thus implying that the signaling molecule that binds to the receptor and activates GNA1 and GNA3 cannot be the cellulase inducer (Schmoll *et al.* 2009; Seibel *et al.* 2009). The main target of cAMP in this signaling cascade is cAMP dependent protein kinase A (PKA), which – after binding of cAMP to the regulatory subunit that inhibits the activity of PKA – dissociates from it and phosphorylates its target proteins. Schuster *et al*. have shown that the adenylate cyclase of *T. reesei* is required for cellulase induction, because disruption of the gene encoding the catalytic subunit strongly impairs cellulose gene transcription (Schuster *et al.* 2012). Interestingly, however, protein kinase A (PKA1) appears to influence cellulase gene transcription negatively because a knock-out in the *pka1* gene actually increases *cel7A* and *cel6A* expression. Thus the positive effect of cyclic AMP must act via another downstream player, which remains to be identified, and PKA1 may be involved via yet another mechanism.

Engineering the lactose catabolic pathway

Induction of cellulases is – beside their natural substrate cellulose and its derivatives – also accomplished by a number of other inducing carbon sources, among which lactose – a heterodisaccharide (1,4-*O*-β-D-galactopyranosyl-D-glucose), a by-product of the cheese and whey processing industries – is also the most economical one. It induces the whole set of plant cell wall degrading enzymes (Ivanova, Seiboth, Kubicek unpublished results). Its inducing effect is surprising since lactose occurs in nature only in the milk of mammals and is therefore not present in the natural habitat of *T. reesei*. Thus it may rather mimick plant oligosaccharides with b-galactoside configuration that become available during the attack of pre-digested plant biomass. The obvious advantage of lactose over cellulose as carbon sources for fermentations is that it is soluble, an attribute which facilitates the downstream

processing of recombinant proteins, and it is therefore often used as inducing carbon source in combination with the strong cellulase promoters (Harman and Kubicek 1998; Penttilä *et al.* 2004). Although cellulase yields produced on lactose are usually reported to be lower compared to cellulose (Andreotti *et al.* 1980), it should be noted that the highest amount of cellulases produced by the publicly available *T. reesei* strain RUT C30 (Durand *et al.* 1988) was achieved on lactose as a carbon source.

The initial steps in lactose catabolism

Analysis of lactose metabolism in other fungi including the yeast *Kluyveromyces lactis* or *A. nidulans* identified two key players: a lactose permease responsible for its uptake and an intracellular b-galactosidase (GH family 2) for subsequent hydrolysis to D-glucose and D-galactose. In *K. lactis* the *LAC12* permease and *LAC4* β-galactosidase genes share an intergenic promoter region, and a similar situation is found also in other fungi (Fekete *et al.* 2012b) but not in *T. reesei*. Orthologues of these genes are however absent from *T. reesei* and it was so far assumed that lactose is hydrolysed extracellularly by the GH family 35 β-galactosidase BGA1 (EC 3.2.1.23) and other cell-wall bound b-galactosidase activities (Seiboth *et al.* 2005). BGA1 has high galactosyltransferase activity and its galacto-β-D-galactanase activity points to its natural role, the hydrolysis of terminal non-reducing β-D-galactose residues in plant cell wall polymers. Galactitol, formed by the reductive D-galactose catabolic pathway, has been claimed to be the inducer of BGA1 expression during growth on lactose (Fekete *et al.* 2007). However, a genome-wide transcriptional analysis identified recently a novel lactose permease, whose knock out completely abolishes growth on lactose (Ivanova, Seiboth, Kubicek unpublished results). The further fate of the so taken up lactose is unclear, since a candidate for an intracellular β-galactosidase activity is still missing.

Intracellular D-galactose utilization

As a result of the extracellular lactose hydrolysis, D-galactose and d-glucose are taken up and are further assimilated. We will focus our attention on β-galactose catabolism as D-glucose does not induce cellulases even at low growth rates whereas D-galactose is able to induce

cellulases (see below). A key for the understanding of the further catabolism is found in the stereospecificity of the D-galactopyranose released from lactose. D-galactose arising by BGA1 hydrolysis is the found in its β-anomeric form. The b-anomeric D-galactose is usually found in an equilibrium with its a-anomer but the chemical mutarotation necessary for this process is a slow, pH dependent process (Pettersson and Pettersson 2001). Therefore, many organisms enhance this conversion enzymatically by an aldose 1-epimerase (mutarotase). This enzymatic mutarotation is, however, necessary for a fast D-galactose utilization

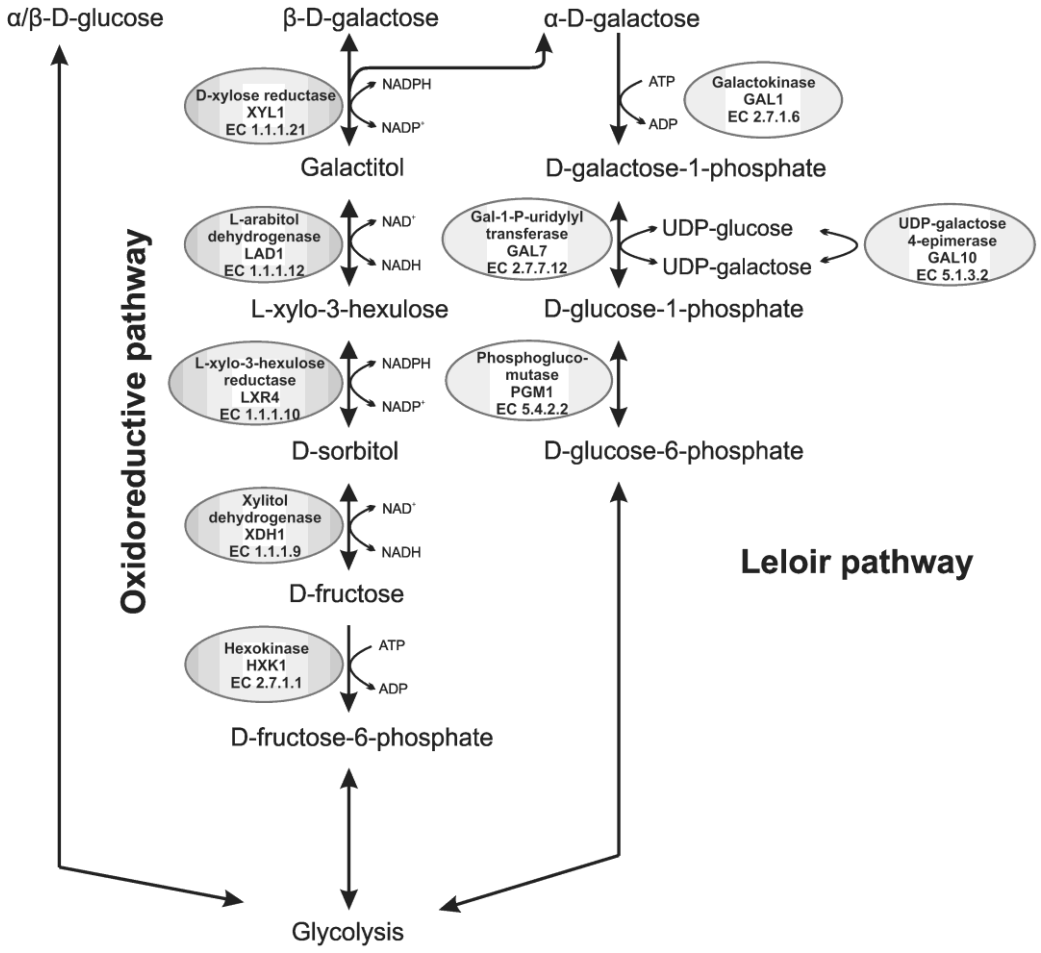

Fig. 4.2 The oxidoreductive pathway of D-xylose and L-arabinose catabolism in fungi

via the canonical Leloir pathway since galactokinase which initiates D-galactose catabolism by C1 phosphorylation is specific for the a-anomeric form (Frey 1996; Holden et al. 2003). Therefore, it is not surprising that many fungi have developed a further possibility to degrade D-galactose independent of its anomeric form. In the case of *T. reesei* or *Aspergillus spp.* this is accomplished by the reductive pathway for D-galactose utilization. In the Leloir

pathway (Fig. 4.2) the sequence of galactokinase (GAL1; EC 2.7.1.6), D-galactose-1-phosphate uridylyl transferase (GAL7; EC 2.7.7.12), UDPgalactose 4-epimerase (UDP-glucose 4-epimerase GAL10; EC 5.1.3.2) and phosphoglucomutase (EC 2.7.5.1) convert α-D-galactose to the glycolytic pathway intermediate d-glucose-6-phosphate (Holden *et al.* 2003). The aldose 1-epimerase (D-galactose mutarotase, EC 5.1.3.3) which provides the galactokinase with the α-D-galactose anomer is especially important for lactose/β-D-galactose catabolism
and its absence resulted in a decreased growth rate on lactose in *E. coli* (Bouffard *et al.* 1994). In the yeasts *S. cerevisiae* and *K. lactis* UDP-galactose 4-epimerase and aldose 1-epimerase are combined to a single bifunctional protein Gal10p whereas the GAL10 of *T. reesei* and other fungal orthologues lack this C-terminal aldose 1-epimerase domain. The genome of *T. reesei* contains three putative aldose 1-epimerase encoding genes (*aep1-3*), however, none of these genes was expressed during growth on lactose and a mutarotase activity was absent during growth on lactose (Fekete *et al.* 2008b). Expression of the C-terminal aldose 1-epimerase part of the *S. cerevisiae* Gal10 in *T. reesei* increased growth on lactose, which implies that the enzymatically catalyzed β-D-galactose mutarotation is either absent or inefficient and that the operation of the Leloir pathway for d-galactose during growth on lactose depends mainly on chemical mutarotation. Other important difference to the model systems of *S. cerevisiae* and *K. lactis* are found in the physiological role of the Leloir pathway and its regulation.

The *S. cerevisiae GAL* regulon is a eukaryotic model systems for transcriptional regulation in which induction of the *GAL* genes is controlled by the interplay of the transcriptional activator, Gal4p, a repressor, Gal80p, and an inducer, Gal3p (Bhat and Murthy 2001; Sellick *et al.* 2008). Gal3p is a paralogue of the galactokinase Gal1p, but misses its galactokinase activity. Regulation of transcriptional induction is similar
in *K. lactis* (Rubio-Texeira 2005) with a number of differences including the combination of the inducer (Gal3p) and galactokinase (Gal1p) function in a single Gal1p protein. Similarly, *T. reesei* and other fungi miss a Gal3p protein. Expression of the *gal* genes is found in *T. reesei* under non-inducing and d-glucose repressing conditions, and *gal1* and *gal7* can be further induced (about two- to threefold) by D-galactose and L-arabinose (Seiboth *et al.* 2002a; Seiboth *et al.* 2002b; Seiboth *et al.* 2004). Regulation of the other Leloir pathway genes is, however, completely independent of the presence of the galactokinase protein or its activity (Hartl *et al.* 2007) and an orthologue of the transcriptional activator Gal4p is absent.

Another difference to the two model yeasts is that *T. reesei* strains deleted in the galactokinase encoding gene *gal1* were still capable of growing on d-galactose and this growth was not due to a residual galactokinase activity (Seiboth *et al.* 2004). A first hint towards this second pathway was obtained by the transient accumulation of galactitol during growth of a *T. reesei gal1* knockout strain on lactose which (Seiboth *et al.* 2004). This second D-galactose pathway is initiated by the D-xylose reductase XYL1 (EC 1.1.1.21) (Seiboth *et al.* 2007a) which catalyzes the NADPH dependent reduction of D-galactose (beside D-xylose and L-arabinose) to galactitol. The second step in this pathway, *i.e.* the NAD^+ dependent oxidation of galactitol is catalysed by the *T. reesei* L-arabinitol 4-dehydrogenase LAD1. Δ*lad1* strains are unable to grow on galactitol and a simultaneous block of the Leloir pathway at the galactokinase step (Δ*lad1*Δ*gal1*) led to the inability of this strain to grow on d-galactose at all. LAD1 was actually the first identified enzyme of this second D-galactose pathway (Seiboth *et al.* 2004). Characterization of the recombinant produced LAD1 led to the unexpected finding that galactitol is oxidized to L-xylo-3-hexulose (Pail *et al.* 2004). The enzyme acting on this uncommon sugar has recently been identified in *T. reesei* as the NADPH-dependent L-xylo-3-hexulose reductase LXR4 (Metz, B. and Seiboth, B., unpublished data). L-xylo-3-hexulose is converted to sorbitol and finally oxidized by another NAD-dependent polyol dehydrogenase, XDH1 (termed xylitol dehydrogenase) to D-fructose. The latter is phosphorylated by hexokinase to fructose-6-phosphate, an intermediate of glycolysis.

The importance of this oxidoreductive pathway has been demonstrated in the catabolism of lactose in *T. reesei* (Seiboth *et al.* 2007a). Despite that β-D-galactose generated from lactose must be catabolised via the reductive galactose catabolizing pathway, disruption of the *gal1* gene still results in a decrease in growth rate on lactose (Seiboth *et al.* 2004) and overexpression of an aldose-1 epimerase improved the growth rate on lactose (Fekete *et al.* 2008b). Mutants in the aldose reductase XYL1 display a strongly reduced growth on lactose. This pathway has also been termed a “stowaway shunt” because the oxidoreductive enzymes involved are the same that catalyze the catabolism of L-arabinose in *T. reesei*.

The physiological relevance of this pathway for D-galactose utilization seems to differ between fungi. In *A. nidulans* the pathway can fully compensate for the loss of the Leloir pathway (Roberts 1970; Fekete *et al.* 2004) while in *T. reesei* inactivation of the Leloir pathway leads already to strains which are significantly impaired in their growth on D-

galactose. One reason for this might be found in the fact that the enzymes of this pathway are adapted for their role in pentose catabolic pathways and their expression is in general lower on d-galactose than on the pentose sugars. For *A. niger* it was recently shown that at least some of the steps are catalyzed by more specific enzymes which are not involved in the degradation of L-arabinose (Koivistoinen *et al.* 2012; Mojzita *et al.* 2012b). The major importance of this pathway is, however, found in its role in β-D-galactose and lactose catabolism by the formation of the inducer galactitol for b-galactosidase BGA1 (Fekete *et al.* 2007) and its importance for cellulase induction.

Metabolic engineering of cellulase gene expression on lactose

Cellulase expression during growth on lactose is a growth rate dependent process with highest cellulase production during low growth rates (Pakula *et al.* 2005; Karaffa *et al.* 2006). The rate of the extracellular lactose hydrolysis is therefore critical for cellulase gene expression. A lowering of its rate by deletion of the *bga1* gene in *T. reesei* encoding the major extracellular β-galactosidase BGA1 affected mainly growth on lactose but not cellulase expression. However, the constitutive overexpression of *bga1* led to higher growth rates on lactose and significantly inhibited cellulase expression during growth on lactose (Seiboth *et al.* 2005). The extracellular lactose hydrolysis leads to an interesting question: are its monomers also able to induce cellulases? During normal growth on these carbon sources neither D-glucose nor D-galactose nor mixtures of them are able to induce cellulase transcription, even in a carbon catabolite derepressed *cre1*-negative background (Seiboth *et al.* 2004). However, growth on D-glucose and D-galactose is much fast than on lactose as carbon source. By using defined growth rates in carbon limited chemostat cultivations some cellulase induction by D-galactose and a mixture of D-galactose and D-glucose occurred at low growth rates. No induction was apparent under the same conditions with D-glucose as limiting carbon source. Nevertheless, cellulose expression under these conditions was significantly lower then found during growth on lactose at the same low growth rate, thus implying that lactose is still a superior inducer that a slow metabolism of D-galactose alone is not sufficient to account for the efficient cellulase induction. An equimolar mixture of D-galactose and galactitol, also led to cellulase induction at higher growth rates but still did not reach the levels obtained for lactose (Karaffa *et al.* 2006). In the absence of a pure b-anomer

the experiments described above were performed with D-galactose which is a mixture of the α- and β-anomer. Since enzymatically catalysed mutarotation of β-D-galactose is either absent or inefficient in *T. reesei* we investigated if the availability of β-D-galactose arising from lactose may thus be a relevant parameter in the induction of cellulase gene expression. Strains which expressed the C-terminal aldose 1-epimerase domain of the *S. cerevisiae* Gal10p showed a moderate increased growth rate on lactose but also led to a significant down-regulation of cellulase gene transcription. While this points towards a prominent role of the β-D-galactose anomer in cellulase induction by lactose, the actual mechanism must be more complex: despite of that D-galactose generated from lactose will be mainly catabolized via the second d-galactose pathway, deletion of the *gal1* gene results also in a strong decrease of cellulase formation on lactose (Seiboth *et al.* 2004). In *S. cerevisiae* the inactive galactokinase Gal3p acts as a coactivator for the induction of the *GAL* genes (Bhat and Murthy 2001; Sellick *et al.* 2008) and is needed for the fast induction of the *GAL* genes. In these Δ*gal1* strains, cellulose induction can be restored by retransformation with the structurally unrelated galactokinase gene from *E. coli* but cannot be restored by the introduction of an enzymatically inactive *T. reesei* GAL1 (Hartl *et al.* 2007). Therefore, galactokinase activity itself is important for cellulase induction by lactose. Inactivation of the subsequent step in the Leloir pathway, in which the *T. reesei* GAL7 converts D-galactose-1-phosphate into UDP-galactose, has no negative effect on cellulase induction except that the cellulose transcripts have a longer half-life (Seiboth *et al.* 2002a). So in contrast to the regulation of the *gal* genes (which showed that galactokinase is dispensable for *gal7* induction), transcription of the cellulases genes by lactose requires galactokinase activity. Similar to the situation in the Leloir pathway deletion of later steps did not affect cellulase expression, but affected growth on lactose. A *xyl1/gal1*-double knock-out did not display an additive action but exhibited an expression level similar to that of the *gal1* knock-out, which would indicate that a blockage in either of the two pathways acts on the same target (Hartl *et al.* 2007). At the moment different models exist to explain the effects of the engineering of the galactose pathway. One explanation would be that an inducer is formed during lactose catabolism and that this oligosaccharide is composed of metabolites of both from the Leloir and the second pathway. A metabolomic analysis of intracellular oligosaccharides formed in *T. reesei* showed that several galacto-oligosaccharides were detected, whose intracellular concentrations changed in a consistent way with cellulase formation (our

unpublished results). Another possibility is that the interruption of one of the two pathways interferes with induction because of accumulation of a metabolite that would disturb the metabolic balance. Further investigations in this area are therefore clearly needed to clarify this point.

Engineering the pentose catabolic pathways for D-xylose and L-arabinose

In contrast to cellulose, the hemicelluloses are composed of monomers that serve as inducing carbon sources for different hemicellulases. The fact that some of them (D-xylose, D-mannose and D-galactose) can exert carbon catabolite repression at high concentrations (Aro *et al.* 2005; Mach-Aigner *et al.* 2010) does not interfere with this assumption because they may naturally not accumulate in the medium at concentrations high enough to cause this effect. Also disaccharides (xylobiose, sophorose, glucose-β-1,2-D-xylose, lactose) have been reported to induce xylanases and other hemicellulases. The effect of xylobiose may be due to the formation of D-xylose at a slow rate by the action of β-xylosidase. Likewise, L-arabinose and L-arabitol have been shown to induce expression of the genes encoding enzymes involved in the degradation of arabinans (Aro *et al.* 2005).

The interconnected pathway for D-xylose and L-arabinose utilization

The fungal oxidoreductive catabolism of these two pentoses is unique for fungi and produces ultimately D-xylulose 5-phosphate, which is an intermediate of the canonical pentose phosphate pathway (Fig. 4.3). In contrast, prokaryotes use an isomerase step to convert D-xylose to D-xylulose, and L-arabinose to L-ribulose. D-xylulose 5-phosphate is then either formed by the action of xylulokinase (in the case of D-xylose) or a sequence of L-ribulokinase and L-ribulose-5-phosphate 4-epimerase (in the case of L-arabinose) (Mishra and Singh 1993).

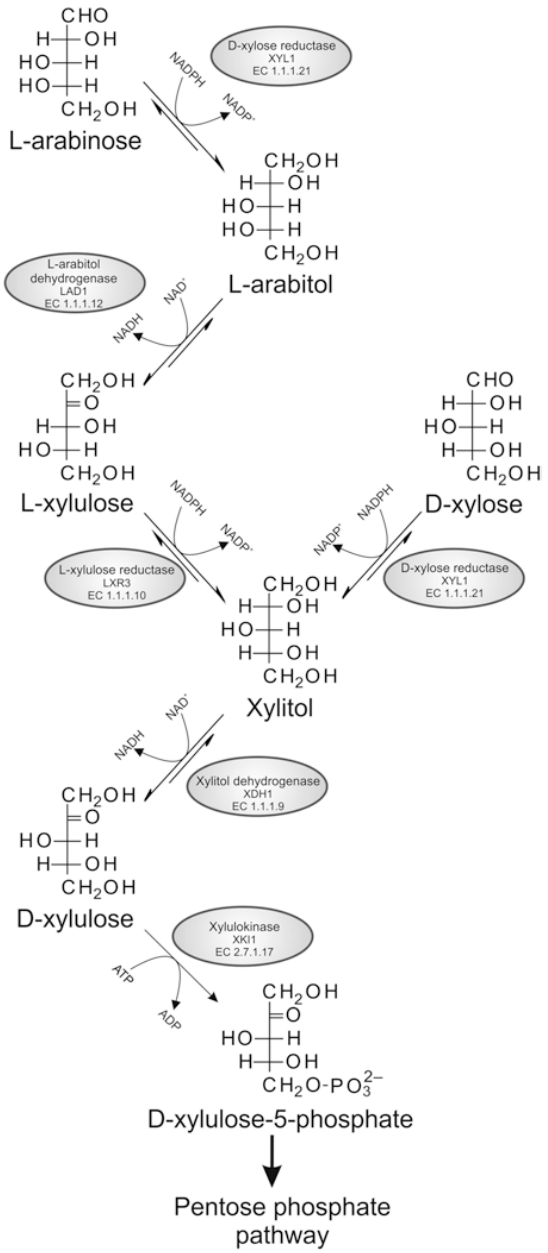

Fig. 4.3 Metabolic pathways involved in the degradation of lactose by *T. reesei*

This fungal oxidoreductive pathway for pentose catabolism consists of a series of reactions in which an NADPH dependent reduction is followed by an NAD^+ dependent oxidation. Thus, although the overall redox balance of these two pathways is neutral, yet it results in an imbalance of the NAD^+/NADH and $NADP^+$/NADPH ratios. The pathways for D-xylose and L-arabinose catabolism are interconnected and share two enzymes, *i.e*. xylitol dehydrogenase and the xylulokinase. The enzyme(s) that catalyze(s) the first NADPH-dependent reduction of L-arabinose and D-xylose differs depending on the fungus: gene deletion analysis of *T. reesei xyl1* encoding the D-xylose reductase XYL1 shows that this enzyme is responsible for both D-

xylose and L-arabinose catabolism because its absence results in significantly impaired growth and reductase activity on both pentoses (Seiboth *et al.* 2007b; Akel *et al.* 2009). In *A. niger*, an additional reductase LarA was identified with a preferred specificity for L-arabinose and whose absence significantly reduced growth on L-arabinose but not D-xylose (Mojzita *et al.* 2010a). The xylitol dehydrogenase XYL1 is the second enzyme of D-xylose pathway and the first enzyme of the common part of the pathway with L-arabinose.

Genetic evidence showed that xylitol dehydrogenase is not essential for D-xylose and xylitol catabolism and that another polyol dehydrogenase, L-arabinitol 4-dehydrogenase LAD1, can partially compensate for its loss (Seiboth *et al.* 2003). However deletion of both *lad1* and *xdh1* in *T. reesei* fully impairs growth on D-xylose and xylitol. LAD1 is also the second enzyme in L-arabinose catabolism following XYL1 and is essential for L-arabinose and L-arabitol catabolism (Pail *et al.* 2004). Identification of L-xylulose reductase (LXR), the enzyme that catalyzes the third step, proved to be a difficult task due to presence of many potential candidates with L-xylulose reductase activity in the fungal genomes. The first gene that encoded a fungal enzyme with L-xylulose reductase activity (LXR1) was identified in *T. reesei* (Richard et al. 2002) but subsequently demonstrated to actually be a D-mannitol dehydrogenase that is involved in developmental processes (Metz *et al.* 2009). Only recently, an NADPH-dependent L-xylulose reductase (LXR3) could be identified in *T. reesei*, whose deletion results in strongly reduced growth on L-arabinose and L-arabinitol (Metz, Kubicek and Seiboth, unpublished data). Phosphorylation of D-xylulose at C-5 is catalyzed by xylulokinase, an enzyme present in *T. reesei*. Its functional characterization in *A. niger* showed that it is essential for L-arabinose and D-xylose catabolism.

Engineering the pentose catabolic pathway for hemicellulase overexpression

T. reesei contains three GH11 (XYN1, XYN2 and XYN5), one GH10 (XYN3) and at least one GH30 xylanase (XYN4). All but XYN3, which has previously been described to be only expressed upon in a mutant strain (*T. reesei* PC-3-7) on cellulose, L-sorbose and sophorose, are coordinately induced by D-xylose and L-arabinose. Mach-Aigner *et al.* (2011) recently reported that D-xylose does not induce *xyn1* and *xyn2* gene expression in a Δ*xyl1* (xylose reductase) mutant and therefore concluded that a metabolite of xylose catabolism must be the inducer of xylanase gene expression. Unfortunately, they assayed xylanase transcription

essentially after 2 h only, which – as we have shown here – is a too early time point where the real effect of D-xylose is not yet visible. Based on their findings that L-arabitol also induces xylanase gene expression, they concluded that D-xylose may have to be converted to L-arabitol to act as an inducer. However, we show here that addition of D-xylose also resulted in increased xylanase gene expression in a Δ*lxr4* (L-xylulose reductase) mutant, which renders this hypothesis impossible. We have recently investigated the induction of expression of *xyn1, xyn2, xyn4* and *xyn5* by D-xylose and L-arabinose in mutants blocked in individual steps of the oxidoreductive pentose catabolic pathway (Herold *et al.* 2013). Based on the use of the Δ*lxr3* strain, which allows to distinguish between the contribution between the D-xylose and L-arabinose catabolic pathway, as it blocks the interconversion of certain intracellular concentration of D-glucose-6-phosphate resulting from the action of both kinases to stimulate CreA-mediated carbon catabolite repression.

Conclusion

Research with *T. reesei* has strongly benefited from the availability of its genome sequence. Moreover, all the tools needed for systematic and high throughput metabolic pathway manipulation are now available, and can be used to produce regulatory or pathway mutants within a short time. Systems biological approaches (including genomic, transcriptomic, proteomic and metabolomic tools) have already been started towards the identification of alterations that had been introduced into the genomes of high(er) producer strains during classical mutagenesis (Kubicek 2013). In view of the information that is already available on the biochemical pathways and their regulation that lead to the induction of cellulases and hemicellulases, it can be anticipated that systems biological investigation of these engineered strains will be the next step towards a global understanding of the physiology required to form biomass degrading enzymes in *T. reesei*.

Final Discussion

T. reesei is a paradigm for the industrial production of (hemi)cellulolytic enzymes and recombinant proteins and a model organism for the genetics and biochemistry of cellulose and hemicellulose degradation (Keränen and Penttilä 1995; Schuster and Schmoll 2010)Kubicek et al. Biotech for Biofuels. Hemicellulases including xylanases are an important class of enzymes produced by *T. reesei* relevant in diverse industrial processes such as biobleaching or biofuel production. This thesis comprises several aspects of hemicellulose degradation highlighted by the identification of a new xylanase in a genome wide screening of the *T. reesei* genome or the identification of missing links in the catabolism of main hemicellulolytic components including L-arabinose and D-galactose. This work enabled a systematic analysis of the importance of the different catabolic steps for inducer formation for expression of the *T. reesei* xylanases. Beside the well known role of D-xylose for xylanase expression, L-arabinose was identified as a further inducing carbon source for xylanase expression.

Xylanases in T. reesei

The initial breakdown of polymeric xylan is mediated by β-1,4-xylanases. In *T. reesei* four xylanases classified in different glycosyl hydrolase families have been identified. XYN1 and XYN2 of the glycosyl hydrolase family 11 had been studied extensively within the last decades in consideration of their industrial potential (Tenkanen *et al.* 1992; Törrönen *et al.* 1992). In addition the GH10 XYN3 and the GH30 XYN4 have been characterized (Xu *et al.* 1998; Saloheimo *et al.* 2003; Ogasawara *et al.* 2006). XYN4 was only characterized recently and exhibits beside endo- also exo-xylanase activity. In the course of this study a fifth xylanase, the GH11 member XYN5 was identified via genome mining and is suggested to be endo-acting. However, further characterisation of this enzyme will be necessary to define the specific substrate requirements and reaction products of this enzyme. GH families 10 and 11 involve the main amount of xylanases detected in microorganisms and also GH5, GH7, GH8 and GH43 exhibit a xylanolytic active domain but examples for those have been identified in bacteria only (Collins *et al.* 2005). The existence of a putative second GH30 xylanase [Protein ID 69276] in *T. reesei* has been predicted by genomic screening studies but its enzymatic characterization remains pending (Häkkinen *et al.* 2012). Closely related

Trichoderma species such as *T. atroviride* and *T. virens* feature just one GH10 but also four GH11 xylanases (Kubicek 2013). The increased occurence of these xylanase is likely to be the result of gene duplication events. With five genes the Eurotiomycetes *A. niger* (1 GH10, 4 GH11) and *A. nidulans* (3 GH10, 2 GH11) present a similar number of β-1,4-xylanases. *N. crassa* was proposed to have 4 GH10 and 2 GH11 enzymes. Examples for filamentous fungi having a remarkably large number of xylanase genes are *Magnaporthe grisea* and *Stagonospora nodorum* with 10 (5 GH10, 5 GH11) and 14 (7 GH10, 7 GH11) respectively (Sun *et al.* 2012; Kubicek 2013). Xylanase research is focused on the identification of novel xylanases and to broaden understanding of their regulation mechanisms in order to be able to adapt xylanase production for industrial purposes.

Fungal L-arabinose and D-galactose catabolic pathways

Full degradation of hemicelluloses results in the uptake of the different sugar mono- and oligomers. In the end the monosaccharidic sugars are either degraded in the glycolytic pathway (D-glucose and D-mannose) or within other degradation routes (D-xylose, L-arabinose). D-galactose is a special case as it can be converted via the Leloir pathway or an oxidoreductive pathway to a glycolytic substrate. Although most of these catabolic pathways are widely conserved in filamentous fungi, specific adaptations are found and the function and orign of some of the involved enzymes differs in a species dependent manner.

Both D-xylose and L-arabinose are converted to D-xylulose 6-phosphate by an oxidoreductive pathway. They are connected and involve xylitol as the first common intermediate that is further catabolized to D-xylulose. Interestingly there are species specific differences found in the enzymes involved in these reactions. *T. reesei* shares an NADPH dependent reductase XYL1 to target both D-xylose and L-arabinose and XYL1 catalyzes the conversion into their respective polyols (Seiboth *et al.* 2007a; Akel *et al.* 2009). In contrast, *A. niger* makes use of distinct enzymes that are specific for each step of the D-xylose and L-arabinose catabolic pathways. D-xylose is reduced to xylitol by XyrA while L-arabinose reduction to L-arabitol is mediated by LarA. XyrA is upregulated on D-xylose only while LarA is expressed on L-arabinose and initially also on D-xylose but just in the first 4h of incubation on this carbon source. Loss of *larA* leads to impaired growth on L-arabinose while the growth rate on D-xylose remains unaffected. In contrast deletion of *xyrA* causes severe growth defects on D-xylose as sole carbon source (Mojzita *et al.* 2010a).

A further adaption seems to be present in *Magnaporthe oryzae* (Klaubauf *et al.* 2013). Beside a D-xylose reductase a further pentose reductase PRD1 was described which displays activity toward L-arabinose and D-xylose. Phylogenetic analysis shows that PRD1 defines a family of pentose reductases related to fungal D-xylose reductases, but distinct from fungal L-arabinose reductases. Thus in *M. oryzae* this novel reductase might be used for the reduction of D-xylose and L-arabinose respectively.

In *T. reesei* the two dehydrogenases LAD1 and XDH1 have been shown to partly compensate for each other in the degradation of D-xylose and xylitol. Only deletion of both resulted in an inability to grow on D-xylose and xylitol while growth on L-arabinose and L-arabitol is almost absent in a *lad1* deletion strain (Seiboth *et al.* 2003; Pail *et al.* 2004).

In the course of the L-arabinose degradation pathway L-xylulose is formed as a metabolite by reduction of L-arabitol. The specific enzyme that catalyzes this step in *T. reesei* remained unknown for a long time while the other enzymes that are part of the L-arabinose pathway have been identified for *T. reesei* and *A. niger* respectively. Genome wide screening of candidate genes belonging to the short chain dehydrogenase family revealed *lxr3* as the gene encoding the functional L-xylulose reductase during growth on L-arabinose and L-arabitol. LXR3 is, however, phylogenetically different from the *A. niger* L-xyluose reductase LxrA, indicating a separate evolution of these short chain dehydrogenase family members. Both are upregulated on L-arabinose and NADPH dependent but the effect of the inactivation differs in *T. reesei* and *A. niger*. Deletion of *lxr3* leads to a decrease of the total NADPH-specific L-xylulose reductase activity in cell free extracts whereas deletion of *lxrA* in *A. niger* causes total loss of the activity indicating that the *A. niger* counterpart is the only enzyme that exhibits NADPH-dependent L-xylulose reductase activity in this organism. However both mutants show reduced growth on L-arabinose and exhibit no activity with NADH as cofactor (Mojzita *et al.* 2010b).

Interestingly the oxidoreductive pathway of pentose catabolism only involves NADPH dependent reductases and NAD^+ dependent dehydrogenases (Witteveen *et al.* 1989). However, it is possible that also NADH dependent activities contribute in the L-arabinose catabolic pathway and in *A. niger* both NADH and NADPH dependent L-xylulose activities have been described. Deletion of *lxrA* led to complete loss of NADPH dependent L-xylulose activity indicating that it is the only gene expressed during growth on L_arabinose capable of using NADPH as a cofactor for L-xylulose reduction (Mojzita *et al.* 2010b). The overall redox

balance is neutral but the cofactors are not balanced leading to a slower catabolism rate compared to for example glucose catabolism.

Another hemicellulosic compound that is found in nature is D-galactose. Monosaccharidic galactose is released from polysaccharides such as galactoglucomannan, xyloglucan or pectin via β-galactosidases. Beside the well studied Leloir pathway a second oxidoreductive D-galactose catabolic pathway exists in many filamentous fungi. In this oxidoreductive pathway D-galactose is catabolized to D-fructose 6-phosphate via alternating reduction and oxidation steps. The pathway resembles the oxidoreductive D-xylose/L-arabinose catabolic pathway and in *T. reesei* some of the enzymes of these pentose degradation pathways participate in the assimilation of D-galactose. These are the D-xylose/L-arabinose reductase XYL1 which reduces D-galactose to galactitol. LAD1 converts this polyol into L-xylo-3-hexulose and XDH1 targets the metabolite D-sorbitol that is formed from L-xylo-3-hexulose and generates D-fructose within an oxidation reaction (Seiboth *et al.* 2003). As describe above *A. niger* uses distinct enzymes for the reduction of D-xylose (XyrA) and L-arabinose (LarA). However XyrA also exhibits strong activity with D-galactose and thus is supposed to also catalyze the initial reduction step of D-galactose to galactitol (Mojzita *et al.* 2010a). The conversion of galactitol was shown to be catalyzed by LadB in *A. niger* (Mojzita *et al.* 2012b). Both LadA and LadB show similar in vitro activities with galactitol but LadB expression only is induced by D-galactose and galactitol. Furthermore a *ladB* knock-out mutant is unable to grow on galactitol. The product of LAD1 and LadB is in both cases L-xylo-3-hexulose. In both fungi the enzymes that catalyze the further reaction from L-xylo-3-hexulose to D-sorbitol remained unknown for a long time. It was assumed that in analogy with XYL1, LAD1 and XDH1 the *T. reesei* LXR3 would be the candidate for L-xylo-3-hexulose reductase activity but LXR3 does not show activity with L-xylo-3-hexulose. In the course of this work LXR4 in *T. reesei* and XhrA in *A. niger* were identified as the physiologically important L-xylo-3-hexulose reductases. Both short chain reductases are strictly NADPH dependent and the deletion of the genes resulted in inability to grow on galactitol in both fungi. Comparison of the sequences demonstrates that LXR4 is closely related to LxrA and XhrA but more distant to LXR3. This is also reflected by the substrate specificity as LXR3 is restricted to L-xylulose only whereas LxrA exhibits L-xylo-3-hexulose activity and thus can contribute to D-galactose degradation as well. Genes of the canonical Leloir pathway are present in most fungi although this pathway might be not active in all of them. For instance *A. niger* does not grow on D-galactose as sole

carbon source (Fekete *et al.* 2012a; Mojzita *et al.* 2012b). XyrA needs D-xylose as an inducer and after initial conversion of D-galactose to galactitol the latter serves as inducer for the other key players of the pathway such as LadB and XhrA. XhrA homologues can be found at least in *A. oryzae*, *A. fumigates* and *A. nidulans*. However the closest *A. nidulans* XhrA homologue (ANID_03400) was detected at a different genomic locus than the XhrA homologues of the other Aspergilli (chromosome V, contig 2, bases 368108-368936).

Transcriptional induction of β-1,4-xylanases

The well characterized β-1,4-xylanases *xyn1* and *xyn2* were shown to be induced to different extend by diverse carbohydrates. Earlier studies showed that *xyn1* expression is triggered by D-xylose while *xyn2* is rather induced by xylobiose and the cellulose inducing carbohydrates cellulose and sophorose (Zeilinger *et al.* 1996). The lack of induction of *xyn2* by D-xylose is a result of the high levels of D-xylose used in these studies as both xylanases are induced when low levels of D-xylose, the main product of their reaction with xylan, are present in the medium. L-arabitol was proposed as the true inducer of *xyn1* and *xyn2* being formed over the interconnected D-xylose and L-arabinose degradation pathways in the course of D-xylose catabolism (Mach-Aigner *et al.* 2010; Mach-Aigner *et al.* 2011). In addition to *xyn1* and *xyn2, xyn3* was shown to be upregulated in the *T. reesei* mutant PC-3-7 on cellulosic substrates and L-sorbose (Xu *et al.* 1998) but no expression was reported during D-xylose induction. During sporulation xyn2, *xyn3*, the GH30 *xyn4* and the novel identified *xyn5* were induced (Metz *et al.* 2011).

Within this work a systematic study on the regulation was undertaken to unravel the regulation of the complete xylanolytic system of *T. reesei* by the two main xylan pentoses D-xylose and L-arabinose. With the identification of the last missing link of L-arabinose catabolism, LXR3, a set of strains was at hand to study the importance of each step of the catabolism of D-xylose and L-arabinose on the inducer formation for transcriptional activation of these glycoside hydrolase genes. The strain collection comprised strain Δ*xyl1* impaired in D-xylose/L-arabinose reduction, Δ*xdh1* and Δ*xdh1*Δ*lad1* which affect xylitol to D-xylulose formation in both pentose pathways and Δ*lad1* and Δ*xdh1*Δ*lad1* which are unable to convert L-arabitol to L-xylulose respectively in L-arabinose catabolism. Strain Δ*lxr3* is impaired in converting the L-arabinose pathway intermediate L-xylulose to xylitol. Induction studies using 1 mM D-xylose as inducing substance revealed that all five tested xylanases

exhibited transcriptional response to this pentose, with *xyn2* being upregulated remarkably strong and *xyn3* to a very low level though. L-arabinose indeed was successful to trigger xylanase expression as well. In both cases highest induction rates were achieved in the Δ*xyl1* mutant strain suggesting D-xylose and L-arabinose rather than one of their catabolites to act as main inducer. One main finding was that L-arabitol is not the true inducer of xylanase expression formed from D-xylose as postulated earlier (Mach-Aigner *et al.* 2011). This is supported by a number of different observations: (i) We did not find an intracellular accumulation of L-arabitol in the course of our D-xylose induction experiments. (ii) The xylanase expression levels were similar in the Δ*lxr3* and the parental strain pointing out that the partial interruption of the D-xylose and L-arabinose catabolism pathways does not influence inducer formation. (iii) Induction of xylanases by D-xylose was also found in the Δ*xdh1*Δ*lad1* strain which is completely blocked in the step of converting L-xylulose to L-arabitol. Actually induction levels were even higher in this strain. Further experimental evidence supports the conclusion that the inducers of D-xylose and L-arabinose are formed in independent pathways. This is emphasized by the inducing effects in the Δ*lad1* and Δ*xdh1*Δ*lad1* strains which are both impaired in the formation of L-xylulose from L-arabitol. Further investigations pointed out that both D-xylose and L-arabinose can act synergistically rather than antagonistically at least on some of the xylanases (*xyn1, xyn2, xyn4*). Interestingly *xyn5* behaved a bit different in this regard. We did not see a boost in expression when exposed to both D-xylose and L-arabinose at the same time. In fact *xyn5* was induced to low levels by D-xylose in the reference strain and only exhibited stronger expression in the different pentose pathway deletion strains while it showed a strong response to L-arabinose in the reference strain. We propose *xyn5* to react more critically to D-xylose concentration than the other xylanases.

Regulation of xylanase expression

In *T. reesei* the induction of the β-1,4-xylanases by both D-xylose and L-arabinose depends on the transcriptional activator XYR1, a comprehensive regulator of hemicellulase and cellulase expression. Still residual *xyn2* and *xyn4* transcripts were detected in a Δ*xyr1* strain grown on L-arabinose while xylanase formation was totally absent in the Δ*xyr1* strain on D-xylose. Beside direct activation of (hemi)cellulase expression *T. reesei's* XYR1 also regulates different genes of the two pentose catabolic pathways. In Eurotiales the XYR1 ortholog XlnR that is

induced on xylan and D-xylose regulates genes of the xylanolytic system, xylanases and accessory enzyme encoding genes, as well as two cellobiohydrolases and two endoglucanases (van Peij *et al.* 1998; Gielkens *et al.* 1999). In contrast the *M. oryzae* ortholog Xlr1 was recently shown to regulate the pentose catabolic pathway genes but has no influence on hemicellulase expression (Battaglia *et al.* 2013).

In addition a second regulator, AraA that specifically responds to L-arabinose is present in Eurotiales. Due to its high similarity to XlnR, AraR was proposed to have evolved via gene duplication (Battaglia *et al.* 2011b). In *T. reesei* such L-arabinose specific regulator has not been identified yet and there is no AraR ortholog present. Hence in *T. reesei* also the L-arabinose pathway is subject to XYR1 regulation. This assumption is emphasised by the fact that transcriptional activation of *xyl1* which acts as main reductase of both D-xylose and L-arabinose depends on XYR1 (Akel *et al.* 2009). Furthermore also the xylitol dehydrogenase *xdh1* is regulated by XYR1 (Herold *et al.*, unpublished data). Thus XYR1 indirectly regulates the rate of catabolism of the assimilated sugars likely dependent on the amount of inducer present.

In contrast *Aspergilli* involve different enzymes in their pentose catabolism pathways that potentially require more specialized regulators. Thus XlnR is essential for *xyrA* expression on D-xylose. An XlnR multi-copy strain produces increased amounts of XyrA while deficiency of XlnR leads to absence of *xyrA* expression (Hasper *et al.* 2000). However induction of the L-arabinose catabolic genes *larA, ladA* and *lxrA* is not influenced by XlnR (Battaglia *et al.* 2011b). Interestingly AraR exhibits different functions in *A. niger* and *A. nidulans*. In *A. niger* it seems to have a more comprehensive regulation activity as its loss led to severe activity decrease of enzymes involved in L-arabinose catabolism and also *xyrA* and *xdhA* (Battaglia *et al.* 2011a). The *A. niger* AraR regulates the polyol dehydrogenases *ladA* and *xdhA* only. Thus the existence of a second regulator had been proposed for this species to regulate the reductase genes (Battaglia *et al.* 2011b).

In other filamentous fungi the specificities of these regulators are again different. *N. crassa* uses XlnR for the regulation of hemicellulases only while expression of cellulases is controlled over the transcription factors Clr-1 and Clr-2. Homologues of these have been identified in other filamentous fungi but their mechanisms of action are fundamentally different. *A. nidulans* Clr-1 orthologue Clr-B is involved in cellulase regulation only while xylanases are subject to XlnR action (Coradetti *et al.* 2012). Furthermore Clr-B still depends

on the presence of cellulase inducing compounds while ectopic expression of Clr-2 in *N. crassa* led to an inducer independent formation of cellulases which had not been observed for other regulators (Coradetti *et al.* 2013). In *A. oryzae* ManR, that is known to regulate β-mannan catabolism, was identified to be a Clr-2 orthologue (Ogawa *et al.* 2012). Further studies showed that ManR plays a key role in the regulation of cellulolytic genes (Ogawa *et al.* 2013). For *T. reesei* the roles of *clr1* and *clr2* orthologues still have to be investigated.

Xylanase expression is negatively regulated via carbon catabolite repression. In the presence of glucose or even high amounts of D-xylose the wide domain regulator CRE1 is translocated in the nucleus and prevents cellulase and xylanase transcription via direct binding to the promoter regions of the respective genes (Ilmen *et al.* 1996; Vautard-Mey and Fevre 2000; Aro *et al.* 2005). In addition CRE1 is proposed to block the XYR1 binding site and thus inhibits XYR1 action (double-lock mechanism) (Mach *et al.* 1996). In this way, the catabolism of other sugars is repressed and the high energy source glucose is catabolised with a high rate as the preferred carbohydrate. CRE1 is conserved in all filamentous ascomycetes. Its loss had been shown to increased expression of cellulases and xylanases and industrial enzyme overproducing strains such as *T. reesei* RUT C30 or RL-P37 make use of carbon catabolite derepression for enhanced enzyme production as they lack a functional *cre1* gene (Ilmen *et al.* 1996). *T. reesei's xyn1* and *xyn2* have been demonstrated to be subject to CRE1 repression (Mach *et al.* 1996; Mach-Aigner *et al.* 2010). Our data show that also the other xylanases *xyn3, xyn4* and *xyn5* are under CRE1 control and thus expressed to a higher extend in a *cre1* knock-out mutant compared to the reference strain in the case D-xylose is present. This finding is consistent with the occurrence of CRE1 binding sites in the promoters of all mentioned xylanase genes. Still *xyn5* appears to be under indirect CRE1 regulation only as it did not get upregulated under derepressing conditions in the absence of an inducer.

With the identification of the novel short chain reductases LXR4 in *T. reesei* and XhrA in *A. niger* that target the D-galactose derived catabolit L-xylo-3-hexulose new possibilities arise to study the regulatory mechanisms of D-galactose catabolism. A positive regulator, GalX is known to be present in most *Aspergilli*, while *A. nidulans* possesses a second regulator that is unique for this species, GalR. Sequence similarity between those genes is pretty low so that evolution of GalR via a duplication event can be excluded. GalR was demonstrated to activate transcription of genes of the Leloir pathway and the oxidoreductive pathway dependent on the presence of inducing compounds such as galactose or galactitol

(Christensen *et al.* 2011). However GalX was shown to regulate the expression of the oxido-reductive pathway genes only and does not influence Leloir pathway gene transcription (Gruben *et al.* 2012). No GalR and GalX homologues are present in *T. reesei* and no other regulators of D-galactose catabolism have been identified in this species to date, hence future research should focus on this task.

In summary the presented studies offer new insights into different aspects regarding hemicellulose degradation and regulation in filamentous fungi, especially *T. reesei*. It is evident from a comparison to other functional studies that fungi have evolved different strategies to regulate the enzymes involved in cellulose and hemicellulose degradation. In addition also the components of the catabolic pathways for D-xylose, L-arabinose and D-galactose degradation differ in fungi. These components have evolved in different ways which results in a high diversity of these pathways. This knowledge also offers an onset for further development of strategies to improve xylanase production in filamentous fungi.

Appendix

Supplemental Material

Chapter 1

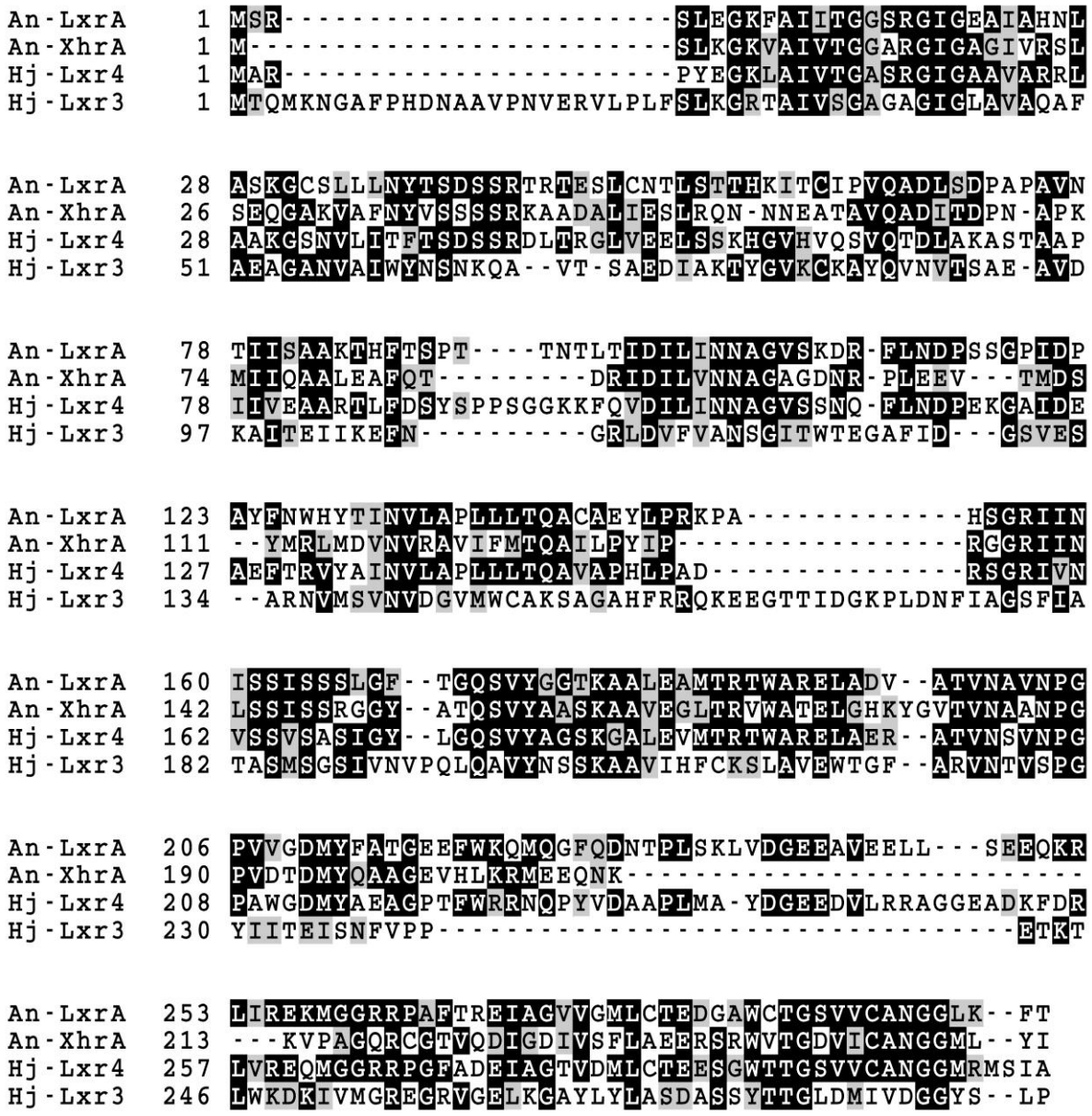

Fig. 1.S1. Protein sequence alignment of LxrA, XhrA, LXR4, and LXR3. In *Aspergillus niger*, two close homologues LxrA and XhrA function in distinct catabolic pathways for L-arabinose and for D-galactose respectively. In *Hypocrea jecorina* (*Trichoderma reesei*), the close homologue of these two proteins, LXR4, is responsible for L-xylo-3-hexulose conversion to D-sorbitol in the oxido-reductive D-galactose pathway; while more distant homologue, LXR3, carries out the L-xylulose reduction in the L-arabinose catabolic pathway.

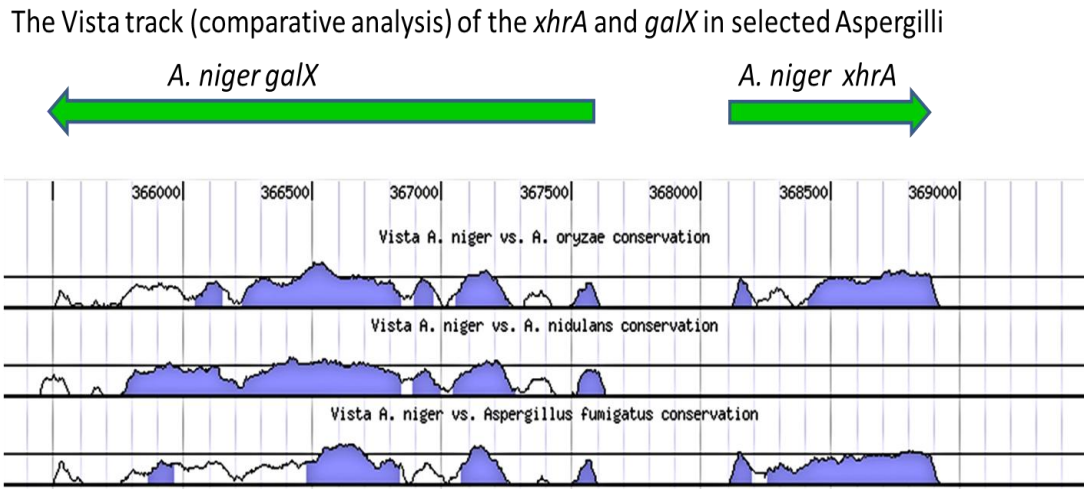

Fig. 1.S2. Comparison of genomic regions covering the *galX* and *xhrA* genes in three *Aspergillus* species with *A. niger*. While all the species contain the *galX* gene, only *A. oryzae* and *A. fumigatus*, but not *A.nidulans*, seem to have the *xhrA* gene (the figure is a modification of a vista visualization obtained from the JGI Aspergillus niger v3.0 web database - http://genome.jgi-psf.org/Aspni5/).

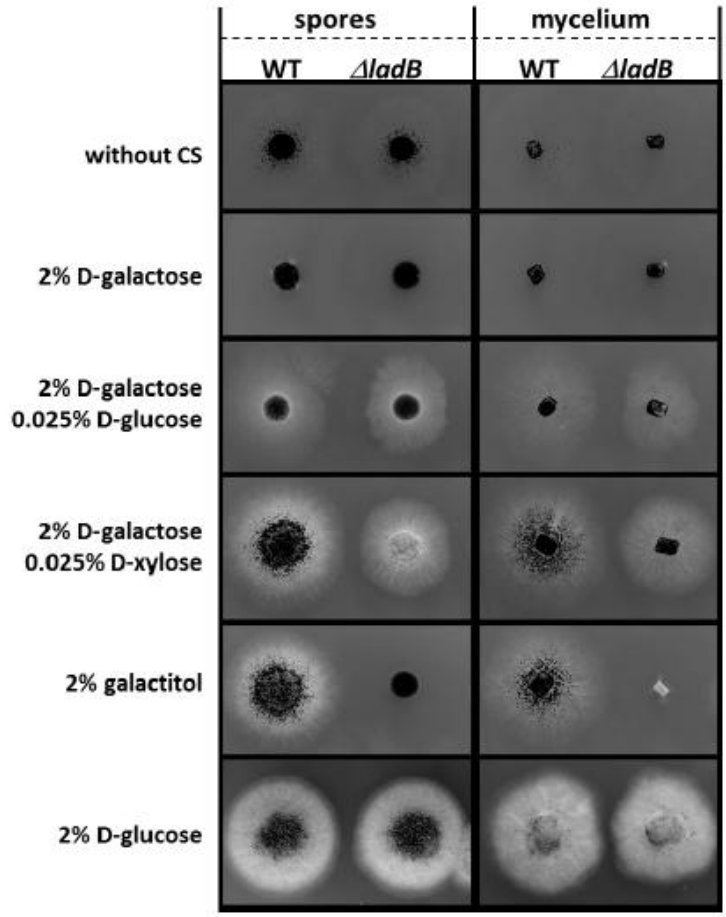

Fig. 1.S3. Growth of the *A. niger* strain ATCC 1015 (WT) and the ATCC 1015 with a deletion in *ladB* (Δ*ladB*) on different carbon sources (CS). Comparison of the growth originated from either conidiospores (leftpanel) or

pre-grown mycelium (right panel). The conidiospores were collected from cultures grown on PDA (potato-dextrose agar) plates and kept at -80°C. The mycelia were produced by inoculating the spores into YESG (2% yeast extract; 4% sucrose; 3% gelatin) medium and cultivating at 28°C for 18 hours. The mycelium was collected by filtration, washed with sterile water, and cut into identical pieces prior to the inoculation on the agar plates. The test plates contained YNB (yeast nitrogen base), 2% agar, and a carbon source as indicated in the figure. The cultivation was carried out at 28 °C for 4 days in the case of spore-inoculation or 3 days in the case of mycelium-inoculation.

Chapter 2

Table 2.S1. Summary of the analysis of twenty LXR candidates of the 117 SDRs of the *T. reesei* genome against the NCBI database, the NCBI *T. reesei* EST database and the *Candida guilliermondi* database.

Protein ID	NCBI (BLASTP	*T. reesei* ESTs	*C. guillermondi*	RT PCR		
				Gal	Ara	Glu
123265	+	+	+	-	-	-
81553	+	+	+	+/-	+/-	-
52718	+	+	+	-	-	-
77202	+	+	+	-	-	-
65433	+	+	+	-	-	-
46936	+	+	+	+	+	++
108201	+	+	+	-	-	-
22512	+	+	+	-	-	-
76114	+	+	+	-	-	-
69840	-	-	+	-	-	-
54086	+	-	+	++	++	+
120288	+	-	+	-	-	-
60033 *(lxr3)*	+	-	+	+++	+++	+/-
65588	+	-	+	-	-	-
62439	+	-	+	+	+	+
66175	+	-	+	-	-	-
122079	+	+	+	+/-	+++	+/-
123553	-	+	+	-	-	-
69502	+	-	+	-	-	-
54991	+	-	+	+/-	+/-	+/-

*Sequences were analyzed by BLASTP against the NCBI database (+ corresponds to e value <$1e^{-80}$) or *C. guilliermondi* genome database (+corresponds to e value <10^{-30}) or for the presence (+) or absence (-) in the NCBI EST database of *T. reesei*. Expression was tested by RT PCR after replacement to the indicated carbon source (1 %; Ara, L-arabinose; D-galactose, Gal; and D-glucose, Glu) after 4 hours. Results indicated the relative level of transcription from high (+++) to absent (-).

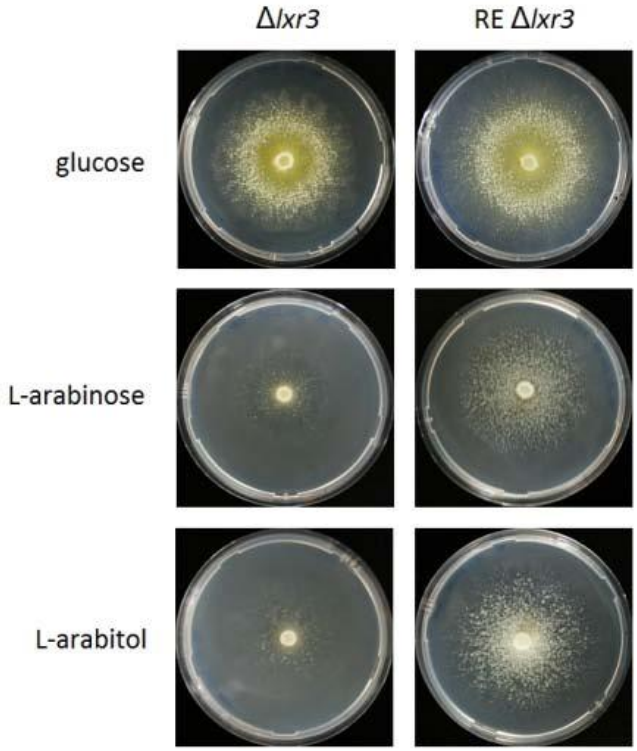

Fig. 2.S1. Growth test of *T. reesei* Δ*lxr3* and RE Δ*lxr3,* a Δ*lxr3* strain retransformed with *lxr3* on agar plates. Both strains were grown under the same conditions as in Fig. 2.3 on different carbon sources (1 % w/v) for 3 d.

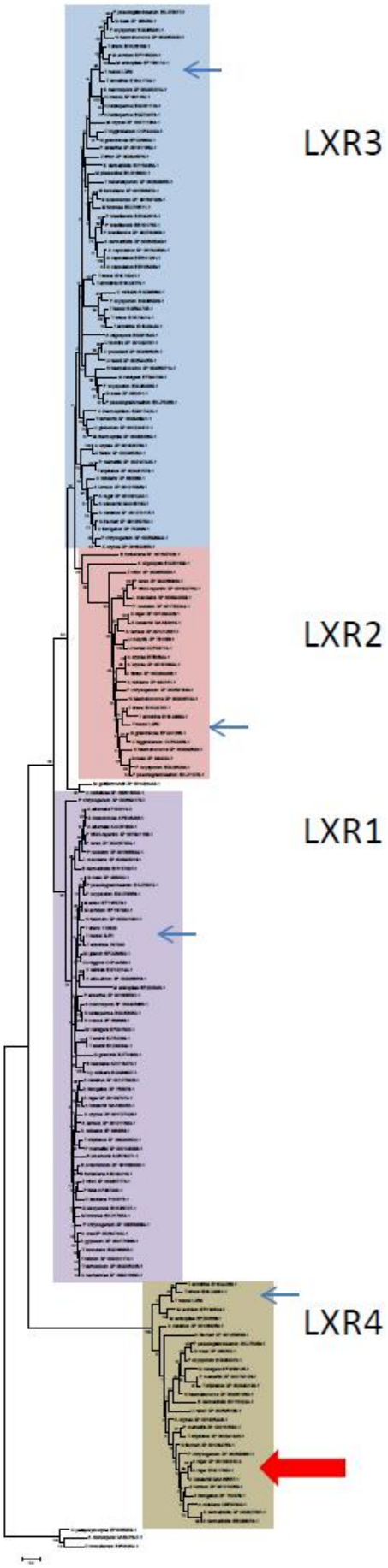
LXR3
LXR2
LXR1
LXR4

Fig. 2.S2. Phylogenetic analysis of L-xylulose reductases and related proteins. The four *T. reesei* LXR proteins LXR1 (D-mannitol 2-dehydrogenase), LXR2, LXR3 (L-xylulose reductase) and LXR4 (L-xylo-3-hexulose reductase), and *A. niger* LxrA were used as a query in a BLASTP search against the NCBI database and subjected to a neighbour joining analysis. Numbers below nodes indicate the bootstrap value. The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions. GenBank Accession numbers of the respective proteins are indicated.

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Curriculum Vitae

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Academic career

12/09 –11/13	Ph.D. studies in molecular biotechnology Institute of Chemical Engineering Vienna University of Technology
10/04 –09/09	Diploma studies in microbiology/genetics Center for Molecular Biology University of Vienna

Education

09/96 –06/04	Secondary School Peraustraße, Villach

Work experience and internships

04/13 – present	Editor Biomedicine/Life Sciences Springer-Verlag GmbH, Vienna
12/09 –12/12	Ph.D. thesis Vienna University of Technology
11/11 –12/11	Internship at the Centro de Investigaciones Biológicas Madrid, Spain
07/11 –08/11	Internship at the VTT Technical Research Centre of Finland Espoo, Finland
04/08 –07/09	Diploma thesis, Plant Vascular Development Gregor Mendel Institute of Molecular Plant Biology GmbH, Vienna
02/08 –03/08	Practical at the Institute for Microbiology and Hygiene Veterinary University Vienna

Participation at international conferences

10/11	Eurofung meeting 2011 Berlin, Germany Talk title: Xylanase induction by D-xylose in *Trichoderma reesei*
04/12	Topical Issues of rational use of natural resources conference St. Peterburg, Russia Talk title: Regulation of xylanase expression in the biomass degrading fungus *Trichoderma reesei*

Publications

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