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Xlr1 is involved in the transcriptional control of the pentose catabolic pathway, but not hemi-cellulolytic enzymes in *Magnaporthe oryzae*

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ABSTRACT

Magnaporthe oryzae is a fungal plant pathogen of many grasses including rice. Since arabinoxylan is one of the major components of the plant cell wall of grasses, *M. oryzae* is likely to degrade this polysaccharide for supporting its growth in infected leaves. D-Xylose is released from arabinoxylan by fungal depolymerising enzymes and catabolized through the pentose pathway. The expression of genes involved in these pathways is under control of the transcriptional activator XlnR/Xlr1, conserved among filamentous ascomycetes. In this study, we identified *M. oryzae* genes involved in the pentose catabolic pathway (PCP) and their function during infection, including the XlnR homolog, *XLR1*, through the phenotypic analysis of targeted null mutants. Growth of the Δ*xlr1* strain was reduced on D-xylose and xylan, but unaffected on L-arabinose and arabinan. A strong reduction of PCP gene expression was observed in the Δ*xlr1* strain on D-xylose and L-arabinose. However, there was no significant difference in xylanolytic and cellulolytic enzyme activities between the Δ*xlr1* mutant and the reference strain. These data demonstrate that *XLR1* encodes the transcriptional activator of the PCP in *M. oryzae*, but does not appear to play a role in the regulation of the (hemi-) cellulolytic system in this fungus. This indicates only partial similarity in function between Xlr1 and *A. niger* XlnR. The deletion mutant of D-xylulose kinase encoding gene (*XKI1*) is clearly unable to grow on either D-xylose or L-arabinose and showed reduced growth on xylitol, L-arabitol and xylan. Δ*xki1* displayed an interesting molecular phenotype as it over-expressed other PCP genes as well as genes encoding (hemi-) cellulolytic enzymes. However, neither Δ*xlr1* nor Δ*xki1* showed significant differences in their pathogeny on rice and barley compared to the wild type, suggesting that D-xylose catabolism is not required for fungal growth in infected leaves.

1. Introduction

The ascomycete *Magnaporthe oryzae* is the major fungal pathogen of rice, but it can also infect other agriculturally important cereals including wheat, rye, barley, and pearl millet (Ou, 1985). The infection of the host plant by *M. oryzae* first involves the penetration of the fungus into the leaf through the cuticle and cell wall using a specialized structure called the appressorium (Wilson and Talbot, 2009). Subsequently, infection hyphae interact with live plant cells during which the fungus spreads into leaf tissues without causing visible damage (1–4 days). This biotrophic phase is followed by a rapid switch to a necrotrophic phase (5–7 days) leading to degradation and destruction of the colonized leaf tissues. During its colonization of the leaf, *M. oryzae* encounters various components of the plant cell wall, some of which can serve as nutrients. The main structural components of the primary cell wall of grasses are arabinoxylan, cellulose and mixed-linked β(1,3),(1,4)-D-glucans (Carpita, 1996; Vogel, 2008). As arabinoxylan represents a significant proportion (10–30% (Deutschmann and Dekker, 2012)) of the cell wall of grasses, the xylanolytic system of *M. oryzae* is likely to be important for its pathogenic lifestyle. Various endoxylanases are secreted during growth on xylan and rice cell walls (Wu et al., 2006, 1997, 1995). Moreover, the *M. oryzae* genome contains a large set of CAZymes involved in arabinoxylan degradation (Battaglia et al., 2011a; Zhao et al., 2013). Deletion of individual components of the xylanolytic system had little effect on pathogenicity (Wu et al., 2006, 1995). However, simultaneous silencing of six

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xylanase encoding genes, has an effect on pathogenicity, leading to a reduction in penetration and wheat leaf colonization rates as well as in symptom development (Nguyen et al., 2011), suggesting that there is functional redundancy among xylanases during infection. Enzymatic degradation of xylan leads to the production of D-xylose and L-arabinose that are metabolized through the pentose catabolic pathway (PCP) in most fungi (Witteveen et al., 1989). This pathway is still uncharacterized in *M. oryzae* except for the recently characterized pentose reductase Prd1, which has been suggested to catalyze the reduction of L-arabinose to L-arabitol in *M. oryzae* (Klaubauf et al., 2013). The expression of some of these genes (xylanases, PCP) is controlled at the transcriptional level by a xylanolytic activator, XlnR, first identified in *Aspergillus niger* (van Peij et al., 1998). Putative orthologs are present in the genome of most filamentous ascomycetes (Battaglia et al., 2011c). In *A. niger* and *Aspergillus nidulans*, the PCP is also regulated by the arabinanolytic regulator AraR, which displays some sequence similarity with XlnR (32% amino acid identity) (Battaglia et al., 2011c). Both XlnR and AraR control the expression of the PCP genes *xhdA* and *xkiA* and have compensatory regulatory functions for these genes (Battaglia et al., 2011b). The other genes of the pathway are either regulated by XlnR (*xyrA*) or AraR (*larA, ladA* and *lxrA*). AraR was only detected in the Eurotiales (e.g. Aspergilli) and likely originated from *xlnR* by duplication (Battaglia et al., 2011c). Therefore, the *M. oryzae* XlnR ortholog, *XLR1*, could potentially control release and catabolism of both D-xylose and L-arabinose.

Here, we identified the PCP genes (Fig. 1) present in the *M. oryzae* genome and show that Xlr1 is the transcriptional activator of the PCP genes during growth on D-xylose but does not appear to affect xylanolytic enzyme activities.

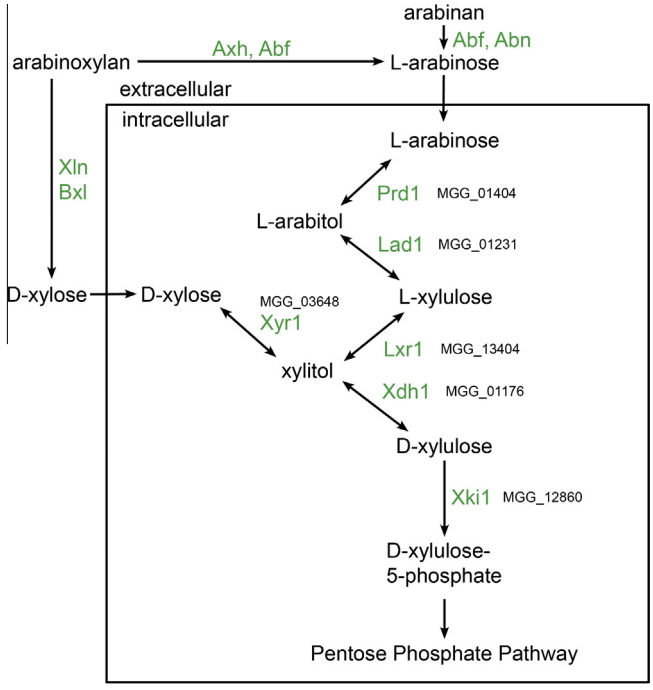

Fig. 1. Schematic representation of the Pentose Catabolic Pathway in *M. oryzae*. Xln = β-1,4-endoxylanase (EC 3.2.1.8), Bxl = β-xylosidase (EC 3.2.1.37), Abf = α-arabinofuranosidase (EC 3.2.1.55), Axh = arabinoxylan arabinofuranohydrolase (EC 3.2.1.55), Abn = endoarabinanase (EC 3.2.1.99), Prd1 = pentose reductase, Lad1 = L-arabitol dehydrogenase (EC 1.1.1.12), Lxr1 = L-xylulose reductase (EC 1.1.1.10), Xyr1 = D-xylose reductase (EC 1.1.1.307), Xdh1 = xylitol dehydrogenase (EC 1.1.1.9), Xki1 = D-xylulose kinase (EC 2.7.1.17). Prd1, Lxr1 and Xyr1 are NADPH/NADP+-dependent. Lad1 and Xdh1 are NADH/NAD+ dependent.

2. Materials and methods

2.1. Strains, media and growth conditions

The wild-type *M. oryzae* strains Guy11 (Silue et al., 1992) and Guy11Δ*ku80* (Villalba et al., 2008) were used in this study. All *M. oryzae* cultures were incubated at 25 °C, *A. niger* strains at 30 °C. TNK-YE (complete medium) was composed of 2 g/l yeast extract, 2 g/l $NaNO_3$, 2 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$ and 0.1 g/l $CaCl_2{\cdot}2H_2O$, 0.004 g/l $FeSO_4{\cdot}7H_2O$ and 1 ml/l of a stock solution of microelements (7.9 g/l $ZnSO_4{\cdot}7H_2O$, 0.6 g/l $CuSO_4{\cdot}5H_2O$, 0.1 g/l H_3BO_3, 0.2 g/l $MnSO_4{\cdot}H_2O$ and 0.14 g/l $NaMoO_4{\cdot}2H_2O$). TNK-MM (minimal medium) is identical to complete medium but yeast extract is replaced by 1 mg/l thiamine and 5 μg/l biotine (Villalba et al., 2008). TNK-YE and TNK-MM were adjusted to pH 5.5–5.8 and carbon sources were added as indicated. TB3 was composed of 20% (w/v) sucrose and 3 g/l yeast extract. For solid media, 1.5% (w/v) agar was added.

Growth tests were performed on TNK-MM (for *M. oryzae*) or Aspergillus Minimal Medium (de Vries et al., 2004) with 25 mM D-glucose, L-arabinose, D-xylose, 1% arabinan, 1% beechwood xylan, 1% birchwood xylan, 1% cellulose and 5 mM D-xylose, 1% wheat bran or 1% rice plant leaves. Strains were inoculated with 2 μl containing 1×10^3 spores or as dilution series (spot inoculation with 1×10^3, 1×10^2 and 1×10^1 spores).

In transfer experiments, *M. oryzae* strains were pre-grown in TNK-YE with 1% D-fructose in a volume of 200 ml in a 1 l Erlenmeyer. Sporulating mycelium of five full grown TNK-YE agar plates was harvested in a total volume of 50 ml H_2O. During harvesting, the mycelium was disrupted into small fragments by scraping with a sterile spatula. Two 200-ml pre-cultures were inoculated per strain using 25 ml of the disrupted mycelium solution. Pre-cultures were cultivated at 120 rpm in a rotary shaker. After 65 h of growth, the mycelium was harvested without suction over a piece of cheese-cloth, washed with TNK-MM (without a carbon source) and 1.5 g mycelium (wet weight) was transferred to a 250 ml Erlenmeyer containing 50 ml TNK-MM with 25 mM D-glucose, L-arabinose or D-xylose. After 2 h of growth the mycelium was harvested with suction over a piece of cheese-cloth, dried between tissue paper and directly frozen in liquid nitrogen. For enzyme assays pre-cultures were transferred to TNK-MM with 1% wheat arabinoxylan (Megazyme, Ireland). Samples of culture supernatant were taken after 24 h, centrifuged and kept on ice until further treatment.

2.2. Sequence analysis

The amino acid sequences of *A. niger* PCP genes were used as queries in a blastp analysis against the *M. oryzae* 70–15 genome sequence (Dean et al., 2005) at www.broadinstitute.org/annotation/genome/magnaporthe_comparative. Amino acid sequences were aligned using MAFFT (http://mafft.cbrc.jp/alignment/software/) L-INS-I (Katoh et al., 2005) and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011). Species used for phylogenetic analysis are listed in Supplemental Table 1.

Molecular weight and isoelectric point were calculated at http://web.expasy.org/compute_pi/.

2.3. Molecular biology methods

Standard molecular methods were used (Sambrook and Russell, 2001). Genomic DNA of *M. oryzae* strain Guy11 was used as a template in PCR reactions. These reactions were carried out using the Phusion High-Fidelity PCR Kit (Finnzymes). The PCR reaction

mixtures contained: 5× Phusion HF buffer, 10 mM dNTPs, 5 μM of each primer, 32 ng gDNA, 3% DMSO, 0.02 U/μl Phusion DNA Polymerase. The following PCR conditions were used: 1 min at 94 °C, 1 min at 56 °C, 5 min at 72 °C for 30 cycles and finally an additional 10 min at 72 °C.

For the *XLR1* deletion construct, the upstream and downstream flank of the *XLR1* gene were amplified by using primer pairs p5.1 (5′-CGTCCCACCGTTTGCATAT-3′) and p.5.2 (5′-CACGGCCTGAGTGG CCCCGATGGAGAGGATTAGAC-3′), and p.3.1 (5′-GTGGGCCATCTAGG CCACTCTATCCTGATGCTTTGG-3′) and p.3.2 (5′-GTGTTGGGATGCTT TGAGG-3′), respectively. The p.5.2 and p.3.1 primers introduced a *Sfi*I recognition sites at the 3′ end of the upstream flank and the 5′ end of the downstream flank, respectively. These *Sfi*I sites were used to combine the upstream and downstream flanking sequences of *XLR1* with the *Sfi*I hygromycin gene fragment derived from plasmid pFP201, generating the *XLR1* deletion cassette. These three fragments, purified from a 0.8% agarose gel with the Nucleospin Extract II kit (Macherey Nagel), were combined using a three point ligation reaction. The mixture for the three point ligation reaction contained 0.2 μg of each of the DNA fragments, 2.5 μl ligation buffer, and 0.5 μl T4 ligase (Fermentas) in a total volume of 10 μl. Subsequently, 5 μl of the ligation mixture was used in a PCR reaction to amplify the three-point ligation product (that is, the *XLR1* deletion cassette) using Accutaq (Sigma) and the primer pair 5′-CGTCCCACCGTTTGCATAT-3′ and 5′-GTGTTGGGATGCTTTGA GG-3′. The amplified and purified *XLR1* deletion cassette was introduced in pGEM-T-Easy (Promega), resulting in plasmid pFP202. For pre-screening of the *M. oryzae* transformants, a PCR was performed using genomic DNA of the transformants and the primers 5′-CGTC CCACCGTTTGCATAT-3′ and 5′-GTGTTGGGATGCTTTGAGG-3′. The hygromycin ORF was amplified using the primers 5′-GGCTTGGCTG GAGCTAGTGG-3′ and 5′-CCCGGTCGGCATCTACTCTA-3′.

The same procedure was used to delete the *XKI1* gene (MGG_ 12860). The left flanking region of *XKI1* was amplified with primers L1XkiF (5′-TTTACCCCGCCATATTCCCG-3′) and LXkiR (5′-CA CGGCCTGAGTGGCCGGGCAGGGTGATACACCGA-3′), while the right flanking region of Xki was amplified with primers RXkiF (5′-GTG GGCCATCTAGGCCTTTCCCAAGTGGCAGTGCAT-3′) and R1XkiR (5′-C AGCAATGTAAGTAGGCAGGC-3′). Primers L2XkiF (5′-GGTGCGGCAA AAAGTCGAAA-3′) and R2XkiR (5′-CCCTTCAAGAAAGCACGTCG-3′) were used to amplify the *xki* deletion cassette from the ligation reaction. The *xki* deletion cassette was cloned in pGEM-T-Easy (Promega), resulting in plasmid pCR1. The screen for *M. oryzae* transformants deleted in *xki* was performed by PCR with the primers F3Xki (5′-TGCGAACTTACCACTGGAGG-3′) and R3Xki (5′-TCTCTC TCTGAGAAGGTTTTGGTG-3′).

2.4. Magnaporthe oryzae transformation

Protoplasts were prepared as described previously (Talbot et al., 1993). Frozen protoplasts were thawed on ice for 20 min and kept on ice during the transformation procedure. The DNA (3 μg) was taken up in a total volume of 40 μl STC (20% (w/v) saccharose, 50 mM Tris (pH 7.5) and 50 mM $CaCl_2{\cdot}2\ H_2O$). Subsequently, 200 μl of the protoplast suspension ($\sim 10^7$ protoplasts) was added to the DNA. The DNA-protoplast suspension was mixed gently by slowly inverting the tube and incubated for 15 min at RT. After 15 min of incubation, 1 ml of PEG 4000 was added to the suspension, mixed gently and incubated for 20 min at RT. Next, 3 ml TB3 was added and the protoplast suspension was incubated on a rotary shaker with gentle agitation (100–120 rpm) for 1–3 h at 26 °C. The protoplasts were collected by centrifugation at 3750 g for 10 min. The supernatant was discarded and the pellet was carefully resuspended in the remaining TB3 (approximately 1 ml). Aliquots of protoplast suspension (0.1–0.3 ml) were transferred to tubes containing 5 ml molten TB3 top agar with hygromycin (240 mg/l). The solution was gently mixed and poured immediately onto regeneration plates (TNK-YE medium with 0.6 M saccharose and 240 mg/l hygromycin) that had been incubated at 37 °C for 20 min. The transformation plates were incubated for 3–7 days at 30 °C.

2.5. Quantitative real-time PCR

Total RNA was extracted from mycelium ground in a micro-dismembrator (B Braun) using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. cDNA was prepared from total RNA (2.5 μg) using Thermoscript RT (Invitrogen) according to the instructions of the manufacturer. The quality of cDNA and relative quantities were assessed on a 1% agarose gel using gel electrophoresis.

The sequences of all primers for qPCR analysis were designed using the Primer Express 3.0 software (Applied Biosystems). The primers were tested to determine the optimal primers concentrations and efficiency. Combinations of the 50 nM, 300 nM and 900 nM (final concentration) per primer pair were tested and based on the dissociation curve the optimal primer concentration per primer pair was set. All primers had between 94% and 103% efficiency and the amplicon length was between 60 and 71 bp. The sequences of the primers and optimal primer concentration are listed in Table 1. qPCR analysis was performed by using the ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, USA) and the ABI Fast SYBR Master Mix (Applied Biosystems, Foster City, USA) according to the instructions of the manufacturer. The amplification reaction was as follows: 95 °C 20 s, 95 °C 3 s and 60 °C 30 s (40 cycles). A dissociation curve was generated at the end to verify that a single product was amplified. Each cDNA was assayed in triplicate in a final volume of 20 μl containing 2 μl of cDNA (diluted 100 fold).

Gene expression is relative to *ILV5* (MGG_01808) expression (Fudal et al., 2007) according to the formula $2^{-(\mathrm{Ct\ gene\ X} - \mathrm{Ct\ ILV5})}$ (Livak and Schmittgen, 2001). Two biological replicates were analyzed. Significance tests were performed with Microsoft Excel. *F*-tests were used to assess differences in variance and two-sample *t*-tests were performed to test for significant differences between the means (fold changes in gene expression normalized to *ILV5*) of wild type (reference) and Δ*xlr1*.

2.6. Enzyme assays

Extracellular enzyme activity was measured using 0.01% *p*-nitrophenol linked substrates, 40 μl of the culture samples, 25 mM sodium acetate pH 5.0 in a total volume of 100 μl. Samples were incubated in microtiter plates for 20 h at 25 °C. Reactions were stopped by addition of 100 μl 0.25 M Na_2CO_3. Absorbance was measured at 405 nm in a microtiter plate reader (FLUOstar OPTIMA, BMG Labtech).

Xylanase activity was determined by measuring the release of reducing groups from xylan. The reaction mixture contained 90 μl of 1% wheat arabinoxylan (Megazyme, Ireland) in 50 mM sodium acetate buffer pH 5, plus 10 μl culture sample. After incubation at 25 °C for 7 h, 150 μl of 3,5-dinitrosalicylic acid (DNS) solution (Miller, 1959) was added and boiled for 10 min at 95 °C. After cooling down, absorbance was measured at 540 nm in a microtiter plate reader (FLUOstar OPTIMA, BMG Labtech). Appropriate controls without either enzyme or substrate were run simultaneously. The quantity of reducing sugar released was calculated from standards of xylose. Absorbance measurements for all assays were performed in triplicate.

Table 1
Primers used in qPCR analysis. nM, primer concentration; fw, forward; rv, reverse.

M. oryzae locus	Gene	Primer (5′ to 3′)		nM
MGG_01404	*PRD1*	fw	CACCCAGAGGGACATTGTTGT	900
		rv	TGACACAGTCGAGGTTCTGCTT	50
MGG_01231	*LAD1*	fw	GGCGGCAAGGTGTTCGT	50
		rv	GCACAGACGCCCTCATGAA	300
MGG_01176	*XDH1*	fw	GGCCAATGGCAAGGTCAAC	50
		rv	GGCCTCTTGGGCCTTGTC	900
MGG_03648	*XYR1*	fw	GCATCGTCGTCACTGCATACTC	900
		rv	GGCGTGCTCCATGTTGAACT	300
MGG_01808	*ILV5*	fw	CCAGCTCTACGACTCGGTCAA	50
		rv	AGTCGGGCTGGCTGTTGTAGT	50

2.7. Pathogenicity assays

Pathogenicity assays were performed using the susceptible rice (*Oryza sativa*) cultivar Maratelli. Rice seedlings were cultivated for 20–30 days at 70% relative humidity and 25 °C day/20 °C night in a phytotronic growth chamber. Fungal spores were harvested from 10- to 14-day old *M. oryzae* cultures. Spray inoculation of rice leaves (3/4 leaves stage) was performed with a spore suspension (3×10^5 spores/ml) containing 0.5% gelatin (w/v). A total volume of 10 ml of spore suspension was used for each pot containing five plants. Inoculated rice plants were first transferred to a humid chamber with 100% humidity at 20–22 °C in darkness for 24 h and then to a greenhouse at 23 °C. Leaf symptoms were recorded 5–10 days after inoculation.

3. Results

3.1. Identification of M. oryzae PCP genes and XLR1

BlastP analysis was performed using the *M. oryzae* 70–15 genome sequence (Dean et al., 2005) and protein sequences encoded by previously identified *A. niger* pentose catabolic genes (de Groot et al., 2007) as queries. Candidate genes are listed in Table 2 and a schematic representation of the pathway is depicted in Fig. 1. No gene homologous to *A. niger larA* (An11g01120) was found in the *M. oryzae* genome. For D-xylose reductase two possible candidate genes were identified (MGG_03648 and MGG_01404). While MGG_03648 is the true ortholog of *A. niger xyrA*, MGG_01404 was shown to encode a novel pentose reductase (PRD1) that can efficiently convert L-arabinose (Klaubauf et al., 2013). MGG_12860 encoding a D-xylulose kinase was shown to be an ortholog of *A. niger xkiA* (Battaglia et al., 2011c).

BlastP analysis using *A. niger* XlnR as a query resulted in two genes: MGG_01414 (bitscore 724, e-value 0.0) and MGG_02880 (bitscore 166, e-value $3e^{-41}$). MGG_01414 showed 49% amino acid identity with *A. niger* XlnR, while MGG_02880 displayed 26% amino acid identity. Phylogenetic analysis of *A. niger* XlnR, MGG_01414 and orthologs from 35 other fungal species showed that MGG_01414 clustered within the group containing the characterized XlnR proteins of *A. niger*, *A. nidulans, Aspergillus oryzae*, *Trichoderma reesei*, *Fusarium oxysporum* and *Neurospora crassa* (Battaglia et al., 2011c) and this gene was therefore named *XLR1*. MGG_02880 does not cluster with XlnR nor AraR (Supplemental Fig. 1) defining a novel family of transcription factor of unknown function.

3.2. Analysis of Δxlr1 and Δxki1 strains

The *XLR1* gene was deleted in the *M. oryzae* Guy11Δ*ku80* strain by replacing the ORF with a hygromycin resistance cassette as described in Materials and Methods. Pre-screening by PCR indicated that one of the transformants contained the *XLR1* deletion cassette and this was confirmed by Southern analysis (results not shown). The reference strain and the Δ*xlr1* mutant were grown on minimal medium media containing as sole carbon source 25 mM of either D-glucose, D-xylose or L-arabinose, and on complex media containing as sole carbon sources either 1% birchwood xylan, beechwood xylan, arabinan, cellulose, wheat bran or rice leaves. Growth of the Δ*xlr1* strain was markedly reduced on D-xylose. A reduction in growth was also observed on birchwood and beechwood xylan although a halo was observed around the colony that was similar in size to the reference strain (Fig. 2), suggesting the production of xylanases is not altered in this mutant. Deletion of the *XLR1* gene did not affect the growth of *M. oryzae* on L-arabinose or arabinan.

Table 2
M. oryzae PCP genes.

Gene	Encoded enzyme	*M. oryzae* locus	Introns[a]	aa[b]	MW[c]	pI[d]	Identity[e]	*A. niger* locus
XYR1	D-xylose reductase	MGG_03648	2	324	36.6	5.4	66%	An01g03740
PRD1	Pentose reductase	MGG_01404	2	328	37.1	6.3	53%	An01g03740
LAD1	L-arabinitol dehydrogenase	MGG_01231	1	372	40.0	5.9	72%	An01g10920
LXR1	L-xylulose reductase	MGG_13404	7	417	44.5	7.3	50%	An08g01930
XDH1	Xylitol dehydrogenase	MGG_01176	1	361	38.1	5.9	61%	An12g00030
XKI1	D-xylulose kinase	MGG_12860	2	577	62.7	5.2	60%	An07g03140
XLR1	Transcriptional regulator	MGG_01414	2	1009	108.9	6.2	49%	An15g05810

[a] Number of introns.
[b] Protein length (amino acids).
[c] Calculated molecular weight (kDa).
[d] Calculated isoelectric point.
[e] Protein sequence identity to *A. niger* homolog.

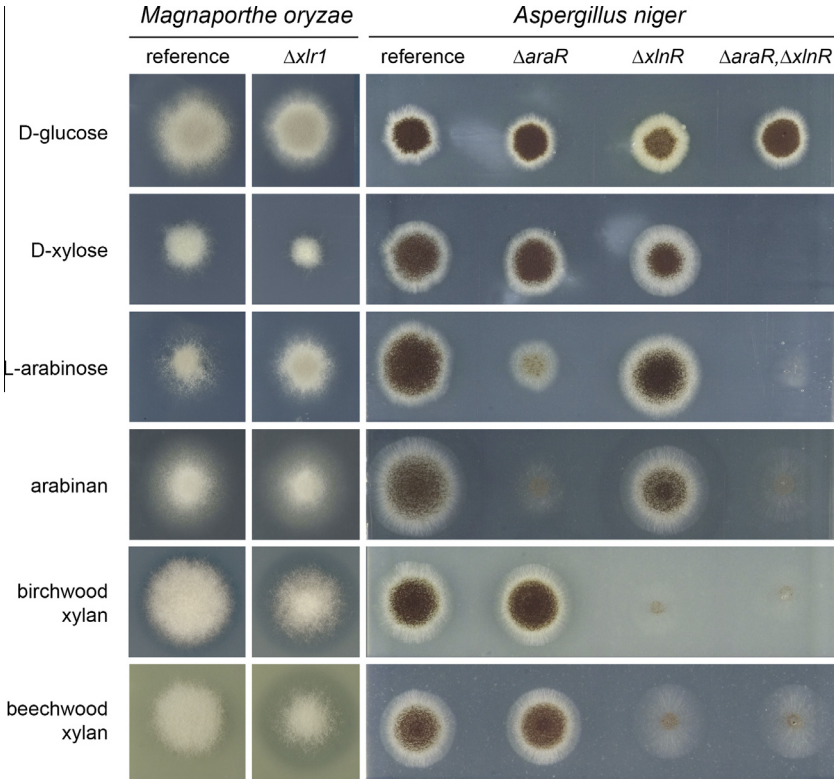

Fig. 2. Growth of *M. oryzae* and *A. niger* wild-type and disruption strains on various carbon sources. The *A. niger* part of the picture was adapted from (Battaglia et al., 2011c).

Δ*xlr1* showed a small reduction in colony size when grown on 1% cellulose +5 mM D-xylose, wheat bran and rice plant leaves (Fig. 3). For comparison, we analyzed the growth of the Δ*xki1* mutant on similar media. Δ*xki1* was unable to grow on D-xylose and L-arabinose and displayed a strongly reduced growth on xylitol, L-arabitol (−95%) as well as a reduction in growth on birchwood xylan (−90%) compared to the reference strain (Fig. 4). A halo around the colony was also observed for Δ*xki1* on birchwood xylan, suggesting that the production of xylanases relative to the growth seems to be similar in mutant and wild type. These phenotypic analyses show that the Δ*xki1* mutant is much more altered in growth on D-xylose and L-arabinose than the Δ*xlr1* mutant.

3.3. Expression levels of PCP genes in wild type and Δxlr1/Δxki1 strains

RNA levels of the PCP genes *PRD1*, *XYR1*, *LAD1* and *XDH1* were analyzed using qRT-PCR. Strains were pre-grown in minimal medium with 1% D-fructose and transferred to 25 mM D-glucose, 25 mM D-xylose or 25 mM L-arabinose for 2 h. All tested PCP genes showed an increase in their expression in the reference strain grown on D-xylose and L-arabinose compared to D-glucose. In addition, all tested PCP genes were reduced in expression in the Δ*xlr1* mutant grown on D-xylose compared to the reference strain. On L-arabinose, expression of *PRD1, XYR1* and *XDH1* was significantly

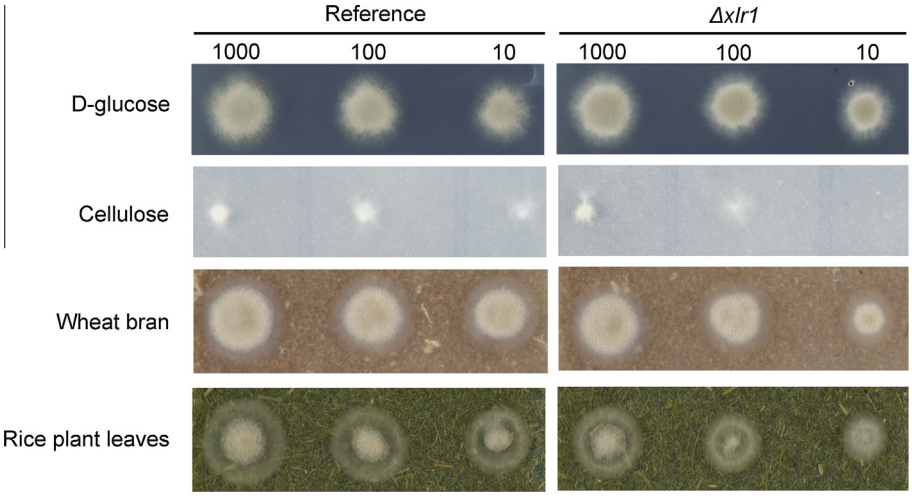

Fig. 3. Growth of *M. oryzae* reference and Δ*xlr1* strain on plant polysaccharides. Dilution series (spot inoculation with 1×10^3, 1×10^2 and 1×10^1 spores) are presented.

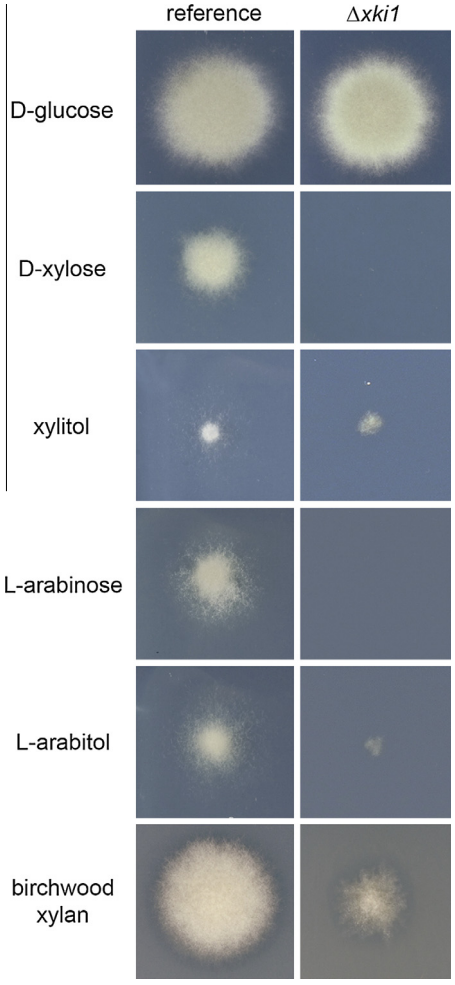

Fig. 4. Growth of *M. oryzae* reference and Δ*xki* strain on various carbon sources.

reduced in the Δ*xlr1* mutant compared to the reference strain, while *LAD1* expression was not affected (Fig. 5). Quantitatively, deletion of *XLR1* had the strongest effect on *XYR1* (D-xylose reductase), since the Δ*xlr1* mutant grown on D-xylose displayed a 30-fold reduction in *XYR1* expression. All tested PCP genes were over-expressed in the Δ*xki1* mutant compared to the reference strain both on D-xylose and L-arabinose (Fig. 5), likely as a compensatory effect.

3.4. Extracellular enzyme activities of Δxlr1 and Δxki1 strains on wheat arabinoxylan

Since both Δ*xlr1* and Δ*xki1* displayed a halo around their colonies on xylan, they likely produce extracellular xylan degrading enzymes. To detect these enzyme activities, pre-cultures of the reference strain, Δ*xlr1* and Δ*xki1* were transferred to TNK-MM with 1% wheat arabinoxylan and supernatants were analyzed after 24 h. Xylanolytic enzymes such as β-xylosidase (BXL), α-arabinofuranosidase (ABF), β-galactosidase (LAC) and α-galactosidase (AGL) as well as the cellulolytic enzymes cellobiohydrolase (CBH) and β-glucosidase (BGL) were assayed. Overall, deletion of *M. oryzae XLR1* does not significantly affect enzyme activities (Fig. 6). Only LAC and to a lesser extent AGL activities were reduced in Δ*xlr1* (60% and 92% of the reference), while the level of activity of the other enzymes was similar to reference strain. Δ*xki1* displayed strong differences in enzyme activities compared to the reference strain. CBH, BGL, BXL and ABF enzymatic activities were increased by 1.5–2-fold in the Δ*xki1* mutant (+160%, +50%, +90% and +20%, respectively), while LAC activity was reduced by more than 2-fold (−60%, Fig. 6). No reduction in xylanase activity was detected in Δ*xlr1* compared to the reference strain (data not shown).

3.5. Pathogenicity assays

Two repetitions of spray inoculations were performed on susceptible rice. Lesions were counted on 30 leaves and analyzed with a global statistical test (Anova). The lesion numbers showed no significant difference between wild type and mutant strains.

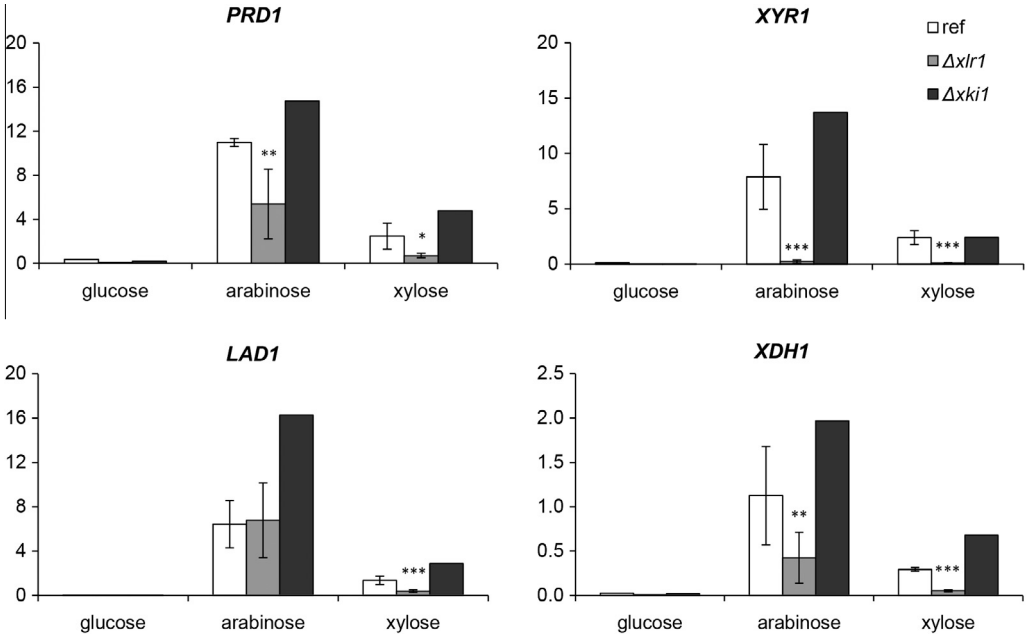

Fig. 5. Expression of PCP genes of *M. oryzae*. Guy11Δ*ku80* (ref), Δ*xlr1* and Δ*xki1* strains were transferred for 2 h to D-glucose, D-xylose or L-arabinose. Quantification of pentose reductase (*PRD1*), L-arabitol dehydrogenase (*LAD1*), xylitol dehydrogenase (*XDH1*), and D-xylose reductase (*XYR1*) mRNA relative to *ILV5* by qRT-PCR. Means and SD (error bars) were calculated from two biological replicates with three technical replicates each. Significance tests were performed between reference and Δ*xlr1*. $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*).

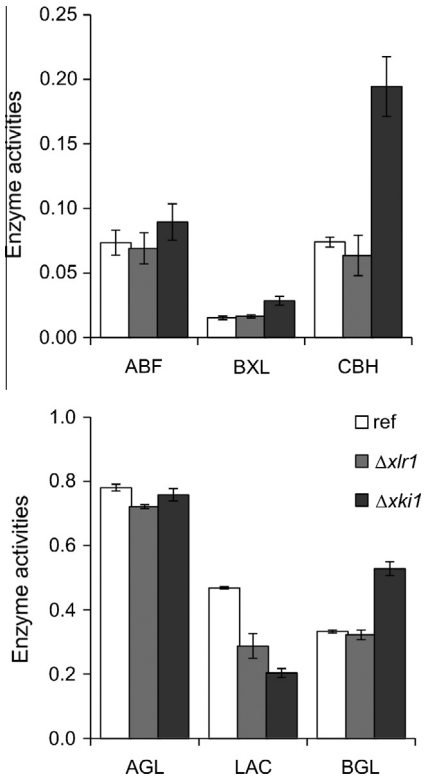

Fig. 6. Comparison of extracellular enzyme activities in reference and disruption strains. Guy11Δ*ku80* (ref), Δ*xlr1* and Δ*xki1* strains were transferred for 24 h to wheat arabinoxylan. Enzyme activities of cellobiohydrolase (CBH), β-xylosidase (BXL), β-glucosidase (BGL), α-arabinofuranosidase (ABF), α-galactosidase (AGL) and β-galactosidase (LAC) in nmol pnp/min/ml. Means and SD (error bars) were calculated from two biological replicates with three technical replicates each.

Furthermore, spot inoculations on detached wounded barley leaves were performed and lesion development was followed for 6 days. No significant effect on lesion area by *Δxlr1*/*Δxki1* strains on wounded barley leaves was observed (Supplemental Fig. 2).

4. Discussion

M. oryzae is a fungal plant pathogen specialized on grasses. Therefore, the pentose catabolism pathway (PCP) could be important for this fungus as the plant cell wall of grasses is considerably composed of arabinoxylan releasing pentoses upon its degradation. So far, little is known about PCP and its regulation in *M. oryzae*. The final step of the PCP is the phosphorylation of D-xylulose, which is catalyzed by D-xylulose kinase. This phosphorylation is absolutely required for the assimilation of arabinoxylan degradation products. As a consequence, *Aspergillus niger* mutants deficient for their D-xylulose kinase are unable to grow on xylose and arabinose as sole carbon source (vanKuyk et al., 2001; Witteveen et al., 1989). We identified a single gene *XKI1* encoding such an enzyme in *M. oryzae* genome and constructed a targeted deletion mutant to assess its role in pentose catabolism and infection. *M. oryzae* Δ*xki1* mutant was unable to grow on D-xylose or L-arabinose and showed a strong reduction in growth on xylitol, L-arabitol and birchwood xylan (Fig. 4), demonstrating the important role of Xki1 for utilizing pentoses. However, this mutant was fully pathogenic on barley (Supplemental Fig. 2), demonstrating that *M. oryzae* does not rely on D-xylose released from the degradation of plant cells walls as a carbon source during infection.

Recently, it was shown that an ortholog of the *A. niger XlnR* gene encoding a transcriptional activator of pentose catabolism genes (*XLR1*) is present in *M. oryzae* (Battaglia et al., 2011c). Therefore, we deleted the *XLR1* gene to assess its role in pentose release from arabinoxylan and its catabolism. Deletion of *XLR1* in *M. oryzae* resulted in a reduced growth (-50%) on D-xylose. This was associated with a strong reduction (29-fold, 3.6-fold, 3.4-fold and 5-fold) in the expression of all the PCP genes (*XYR1*, *PRD1*, *LAD1*, *XDH1*) on D-xylose compared to the reference strain. This strongly suggests that Xlr1 is the transcriptional activator of PCP genes during growth on D-xylose in *M. oryzae*. A strong reduction in growth on D-xylose was also reported for *Hypocrea jecorina* (Stricker et al., 2006) and *Fusarium graminearum* (Brunner et al., 2007) deletion mutants of *XYR1*, the ortholog of *xlnR*/*XLR1*. The residual growth of the *H. jecorina* Δ*xyr1* mutant on D-xylose was hypothesized to result from the functional redundancy of the two reductases, D-xylose reductase and L-arabinose reductase, (Stricker et al., 2006). This explanation seems also plausible for *M. oryzae*, which has a similar phenotype on D-xylose. Prd1 pentose reductase has been suggested to catalyze the first reaction of the L-arabinose pathway in *M. oryzae* (Fig. 1) (Klaubauf et al., 2013). Although *PRD1* expression is reduced in Δ*xlr1* (3.6-fold) grown on D-xylose compared to the reference strain, there is still some expression compared to D-xylose reductase *XYR1* (9-fold higher *PRD1* expression compared to *XYR1*). Prd1 possesses similar affinities for L-arabinose and D-xylose (Klaubauf et al., 2013) and its low expression could compensate for the lack of *XYR1* expression in *M. oryzae*. This functional redundancy in PCP does not exist in *N. crassa* that has no L-arabinose reductase nor Prd1-like pentose reductase. This particularity of the *N. crassa* PCP could explain why its Δ*xlr1* mutant is not able to grow on D-xylose (Sun et al., 2012). On the other hand, *xlnR* deletion mutants of *A. niger* and *A. nidulans* have a growth similar to wild type on D-xylose (Battaglia et al., 2011b). This special case could be explained by the fact that *A. niger xyrA* expression is only reduced by 40% in *ΔxlnR* compared to the reference strain and the corresponding enzymatic activity is still present in *ΔxlnR* (Battaglia et al., 2011c). In addition, AraR can compensate for the loss of XlnR in the regulation of the PCP in *Aspergillus* species (Battaglia et al., 2011c).

Xylanolytic activity is still present and not significantly affected in *M. oryzae* Δ*xlr1*, as evidenced from the halo around the colony on xylans (Fig. 2) and the level of extracellular enzyme activities in its liquid culture (Fig. 6). Therefore, we conclude that Xlr1 does not regulate the xylanolytic system in *M. oryzae*. This finding is surprising since it differs from previous studies in other fungi such as *Aspergillus* species, *H. jecorina*, *Fusarium* species and *N. crassa*, for which the respective XlnR ortholog is the main regulator involved in xylan degradation (Brunner et al., 2007; Calero-Nieto et al., 2007; Mach-Aigner et al., 2008; Marui et al., 2002; Stricker et al., 2006; Sun et al., 2012; Tamayo et al., 2008; van Peij et al., 1998). This result suggests that *M. oryzae* has another regulatory network than Xlr1/XlnR/Xyr1 to control the expression of xylanolytic enzymes. Therefore, we conclude that the function of Xlr1 is only partially similar to its characterized homologs (e.g. *A. niger* XlnR), in that all homologs control D-xylose catabolism, but control of xylanase activity is absent for *M. oryzae* Xlr1.

The limited reduction in growth observed for *M. oryzae* Δ*xlr1* mutant on beechwood and birchwood xylan could be explained by the fact that these complex media contain a high percentage of free D-xylose as well as other sugars (hexuronic acid, L-arabinose, D-glucose and D-galactose (Hespell and Cotta, 1995)) that could serve as alternative carbon sources. Similarly, the growth of *M. oryzae* Δ*xlr1* mutant was only slightly reduced on raw substrates such as wheat bran and rice leaves (Fig. 3). Although wheat bran and cell walls of rice plants are rich in xylan, they also contain other components such as cellulose and proteins. These other components can also serve as carbon sources, which would explain the limited reduction in growth between the reference strain and the Δ*xlr1* mutant.

The growth of the Δ*xlr1* mutant was not affected on L-arabinose and arabinan, indicating that Xlr1 is not involved in L-arabinose release and utilization. However, a significant reduction of the expression of the three tested PCP genes (*XYR1*, *PRD1*, *XDH1*) was observed in the Δ*xlr1* mutant on L-arabinose after two hours, with the most dramatic effect being observed for D-xylose reductase encoding gene (reduction by 35-fold). A similar effect was observed for *XDH1* expression, which was significantly reduced but not abolished in the Δ*xlr1* strain. On the other hand, expression of *LAD1* was not affected and *PRD1* only showed a 2-fold reduction in the Δ*xlr1* strain, with a relatively high residual expression. These results indicate the existence of another factor involved in the regulation of L-arabinose catabolism in *M. oryzae*. Since no ortholog for AraR is present in the *M. oryzae* genome (Battaglia et al., 2011c), an unrelated regulatory protein must be involved in the regulation of the expression of genes involved in L-arabinose catabolism in *M. oryzae*.

The final step of the PCP is the phosphorylation of D-xylulose which is catalyzed by a D-xylulose kinase (vanKuyk et al., 2001; Witteveen et al., 1989). As in *A. niger*, the expression of the *M. oryzae* D-xylulose kinase gene *XKI1* was induced on D-xylose (data not shown). Expression of *xkiA* in *A. niger* is mediated by XlnR as well as AraR, however with a larger influence of AraR (Battaglia et al., 2011c). In *M. oryzae*, *XKI1* expression was reduced by 3-fold in Δ*xlr1* compared to the reference strain on D-xylose (data not shown) suggesting a significant role of Xlr1 in the regulation of *XKI1* expression.

Increased transcript levels of the genes encoding arabinan and xylan degrading enzymes as well as genes for intracellular pentose catabolism (vanKuyk et al., 2001) were observed in the xylulose kinase deficient *A. niger* strain. A correlation with increased levels of L-arabitol suggests that this sugar acts as an inducer of the expression of genes regulated by AraR and XlnR in *A. niger* (Battaglia, 2011 chapter 3; vanKuyk et al., 2001). Based on these findings in *A. niger*, we investigated if *M. oryzae* deletion mutant for *XKI1* was modified for its production of extracellular (hemi-) cellulolytic enzymes and expression of PCP genes. As expected, the *M. oryzae* Δ*xki1* mutant displayed an increase in the expression of all tested PCP genes on D-xylose and L-arabinose (Fig. 5) together with elevated activities of extracellular (hemi-) cellulolytic enzymes. These observations suggest the accumulation of an intracellular inducer in Δ*xki1* as observed in the corresponding *A. niger* mutant. L-arabitol was shown to be the inducer of xylanase expression in *H. jecorina* (Mach-Aigner et al., 2011). Whether L-arabitol or another pathway intermediate is the intracellular low molecular mass inducer in *M. oryzae* xylanase and PCP gene expression needs further investigation. Nevertheless, our results show that extracellular xylanolytic/cellulolytic enzymes as well as PCP genes respond in a similar manner (i.e. over-expression) to the lack of D-xylulose kinase in *M. oryzae* and other fungal species, which points to a conserved regulatory network coordinating the induction of genes encoding sugar-releasing enzymes as well as sugar assimilating enzymes to effectively use extracellularly degraded nutrients. The observation of unaffected xylan degradation in the Δ*xlr1*strain is therefore quite unexpected and suggests a complex network of positive/negative or even compensating regulators involved in the xylanolytic system in *M. oryzae*.

Our initial hypothesis that *M. oryzae XLR1* has a dual function combining those of *Aspergillus* XlnR and AraR, in controlling both the xylanolytic and arabinanolytic system was clearly refuted. Instead Xlr1 is the transcriptional activator of PCP genes, especially of D-xylose reductase encoding gene *XYR1*, but shares the regulation of L-arabinose catabolism with other unknown factor(s). Furthermore, our results indicate significant differences between *M. oryzae* and other fungi studied so far, with respect to regulation of genes involved in xylan degradation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.06.005.

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