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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.

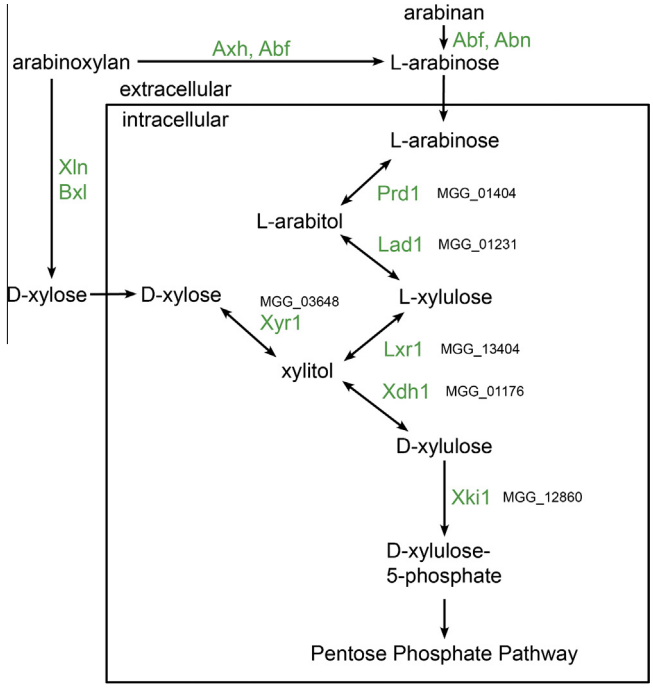

Fig. 1. Schematic representation of the Pentose Catabolic Pathway in *M. oryzae*. Xln = β-1,4-endoxylanase (EC 3.2.1.8), Bxl = β-xylosidase (EC 3.2.1.37), Abf = α-arabinofuranosidase (EC 3.2.1.55), Axh = arabinoxylan arabinofuranohydrolase (EC 3.2.1.55), Abn = endoarabinanase (EC 3.2.1.99), Prd1 = pentose 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