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Research Article

Novel transporters from *Kluyveromyces marxianus* and *Pichia guilliermondii* expressed in *Saccharomyces cerevisiae* enable growth on L-arabinose and D-xylose

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Abstract

Genes encoding L-arabinose transporters in *Kluyveromyces marxianus* and *Pichia guilliermondii* were identified by functional complementation of *Saccharomyces cerevisiae* whose growth on L-arabinose was dependent on a functioning L-arabinose transporter, or by screening a differential display library, respectively. These transporters also transport D-xylose and were designated *KmAXT1* (arabinose–xylose transporter) and *PgAXT1*, respectively. Transport assays using L-arabinose showed that KmAxt1p has K_m 263 mM and V_{max} 57 nM/mg/min, and PgAxt1p has K_m 0.13 mM and V_{max} 18 nM/mg/min. Glucose, galactose and xylose significantly inhibit L-arabinose transport by both transporters. Transport assays using D-xylose showed that KmAxt1p has K_m 27 mM and V_{max} 3.8 nM/mg/min, and PgAxt1p has K_m 65 mM and V_{max} 8.7 nM/mg/min. Neither transporter is capable of recovering growth on glucose or galactose in a *S. cerevisiae* strain deleted for hexose and galactose transporters. Transport kinetics of *S. cerevisiae* Gal2p showed K_m 371 mM and V_{max} 341 nM/mg/min for L-arabinose, and K_m 25 mM and V_{max} 76 nM/mg/min for galactose. Due to the ability of Gal2p and these two newly characterized transporters to transport both L-arabinose and D-xylose, one scenario for the complete usage of biomass-derived pentose sugars would require only the low-affinity, high-throughput transporter Gal2p and one additional high-affinity general pentose transporter, rather than dedicated D-xylose or L-arabinose transporters. Additionally, alignment of these transporters with other characterized pentose transporters provides potential targets for substrate recognition engineering. Accession Nos: *KmAXT1*: GZ791039; *PgAXT1*: GZ791040 **Copyright © 2015 John Wiley & Sons, Ltd.**

Keywords: **arabinose; xylose; transport; yeast; *GAL2***

Introduction

The pentose sugars L-arabinose and D-xylose, referred to as arabinose and xylose without their isomeric descriptors throughout this article, are widely present in nature and their utilization is critical to the economic success of converting lignocellulosic feedstocks to ethanol or hydrocarbon biofuels (Humbird *et al.,* 2011; Menon and Rao, 2012; Davis *et al.,* 2013). The yeast *Saccharomyces cerevisiae*, a well known organism for industrial-scale fermentations, is unable to utilize arabinose or xylose as a carbon source. Previous research to engineer *S. cerevisiae* to ferment arabinose or xylose to ethanol has included the expression of bacterial (Sedlak and Ho, 2001; Becker and Boles, 2003; Karhumaa *et al.,* 2006; Brat *et al.,* 2009; Wisselink *et al.,* 2010; de Figueiredo Vilela *et al.,* 2013; Wang *et al.,* 2013) or fungal (Richard *et al.,* 2003; Bettiga 2009; Bera *et al.,* 2010) pathways and enables *S. cerevisiae* to produce ethanol from arabinose or xylose.

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The first step of pentose conversion is transport into the cell, and this has been identified as a limiting step (Richard *et al.,* 2003; Leandro *et al.,* 2009; Young *et al.,* 2010; Subtil and Boles, 2012). In *S. cerevisiae*, the Gal2 permease has been shown to transport arabinose and xylose (Cirillo, 1968; Becker and Boles, 2003; Sedlak and Ho, 2004; Saloheimo *et al.,* 2007) and, while overexpression was not necessary for growth on arabinose in a previously adapted strain, the growth rate increased with overexpression. The transport rate of arabinose via Gal2p was reported to be 0.32 nM/mg/min at a concentration of 10 mM (Becker and Boles, 2003). Two novel transporters from *Ambrosiozyma monospora*, Lat1p and Lat2p, when expressed in *S. cerevisiae*, have transport rates in 100 mM arabinose of 0.2 and 4 nM/mg/min, respectively (Verho *et al.,* 2011; Londesborough *et al.,* 2014). These rates of transport by the Gal2 permease Lat1p and Lat2p are low in comparison to recently reported rates of transport in two active, high-affinity transport systems from *Kluyveromyces marxianus* and *Pichia guilliermondii* of 20.5 and 16.8 nM/mg/min at 11.8 mM, respectively (Knoshaug *et al.,* 2009) and from the wild-type parent strain *A. monopsora* of 640 nM/mg/min at 100 mM arabinose (Verho *et al.,* 2011).

More examples of the limiting effects of sugar transport on metabolism have been reported for a variety of other sugars. In *S. cerevisiae*, xylose consumption is limited by transport at low concentrations (Gardonyi *et al.,* 2003). Xylose transporters from *Neurospora crassa* and *Pichia stipitis* were identified and overexpressed without an improvement in fermentation rates (Du *et al.,* 2010). In *P. stipitis*, xylose transport has been shown to be limiting in co-fermentation with glucose (Ligthelm *et al.,* 1988), and in *K. lactis* transport is the restrictive step in anaerobic utilization of galactose and raffinose (Goffrini *et al.,* 2002). Fermentation of xylose occurred more rapidly by increasing xylose transport (Runquist *et al.,* 2009) and, through evolutionary engineering, arabinose and xylose transport, were increased, leading to better fermentation (Sanchez *et al.,* 2010). Furthermore, sugar transport rates can direct glucose metabolism via respiration or fermentation in the presence of high external sugar concentrations (Elbing *et al.,* 2004; Otterstedt *et al.,* 2004). These reports show that sugar transport rates can be limiting and, in the case of *S. cerevisiae*, which does not natively utilize arabinose or xylose, may be restricting complete or efficient utilization of pentose substrates and therefore are promising targets for strain improvement.

Earlier investigations of arabinose utilization by non-conventional yeasts focused on fermentation to ethanol (Dien *et al.,* 1996; Kurtzman and Dien, 1998). We previously undertook an extensive study to characterize aerobic growth on arabinose to identify strains that could quickly metabolize large amounts of arabinose, indicating the presence of a high-capacity arabinose transport system and metabolic pathway. Two strains, *K. marxianus* and *P. guilliermondii*, used 20 g/l arabinose within 24 h and appeared to have arabinose transport systems mediated by single, high-affinity, active transporters (Knoshaug *et al.,* 2009). We chose *K. marxianus* as the source strain for cloning of an arabinose transporter via genomic DNA library screening in a strain of *S. cerevisiae* that had been engineered and adapted for growth on arabinose. Unfortunately, *P. guilliermondii* uses an alternative codon for leucine (Tuite and Santos, 1996; Sugita and Nakase, 1999) and would thus not be amenable to transporter cloning through library screening with *S. cerevisiae*, which uses the standard genetic code. We therefore utilized a differential display technique to identify genes that may be involved in arabinose uptake, such that we could codon-optimize and express these putative genes in *S. cerevisiae*. We report here the successful cloning and expression of sugar transporters from *K. marxianus* and *P. guilliermondii* that transport arabinose and xylose when expressed in *S. cerevisiae*. Additionally, we examined the arabinose transport characteristics of Gal2p as a benchmark for these novel transporters.

Materials and methods

Yeast strains, strain cultivation and plasmids

Yeast strains of *S. cerevisiae* and plasmids are listed in Table 1. The yeast *K. marxianus* CBS-1089 was obtained from the Centraalbureau voor Schimmelcultures (CBS) yeast collection and *P. guilliermondii* NRRL Y-2075 was obtained from the Agricultural Research Service (ARS) Northern Regional Research Laboratory (NRRL) collection.

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Table 1. *Saccharomyces cerevisiae* strains and plasmids used in this study

Strain	Genotype	Plasmids
BFY85	*MATα hxt1D::HIS3::hxt4D hxt5D::LEU2 hxt2D::HIS3 hxt3D::LEU2::hxt6 hxt7D::HIS3 gal2D::ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2*	
BFY507	*MAT***a** *ura3-52 trp1-Δ63 his3-Δ200 leu2-Δ1 yhr104w::LEU2* adapted for growth on arabinose	p138, p42
BFY518	Same as BFY507	p138
BFY566	Same as BFY507	p138, p171
BFY583	Same as BFY85	p12
BFY584	Same as BFY85	p42
BFY585	Same as BFY85	p187
BFY590	Same as BFY507; *gal2Δ::HIS3*	p138
BFY596	Same as BFY590	p138, p12
BFY597	Same as BFY590	p138, p42
BFY598	Same as BFY590	p138, p187
BFY605	Same as BFY590	p138, p244
BFY690	Same as BFY85	p244
H2219	*Δhxt1-7 Δgal2 ura3-Δ1::XR/XDH his3-Δ1::XK*	
H2219/p12	Same as H2219	p12
H2219/p42	Same as H2219	p42
H2219/p187	Same as H2219	p187
H2219/p244	Same as H2219	p244
Plasmid	**Marker and expressed genes**	
p12	*URA3*, control vector	
p42	*URA3*, *GAL2* over-expression	
p138	*TRP1*, *B. subtilis* araA, *E. coli* araB, *E. coli* araD	
p171	*HIS3*, 8.8 kb *K. marxianus* genomic DNA fragment	
p187	*URA3*, *KmAXT1* over-expression	
p244	*URA3*, *PgAXT1* over-expression	

Yeast strains were grown in minimal media [0.67% Difco yeast nitrogen base without amino acids (YNB)] containing glucose, arabinose or xylose as the carbon source and 2% agar for solid media. The appropriate amino acids were withheld from the media for plasmid maintenance. Growth conditions were 30 °C with aerobic shaking in liquid cultures. Adaptation for growth on arabinose was performed as described (Becker and Boles, 2003), with the following modifications. Cells were grown in the appropriate selective glucose minimal medium until saturation, then washed and diluted to a starting $OD_{600} = 0.2$ in minimal medium supplemented with 20 g/l arabinose. Cultures were incubated until exponential growth was observed, then diluted twice into the same medium for continued growth to establish the final arabinose-utilizing adapted strain, which was further purified on arabinose streak plates (BFY507). The *GAL2* overexpression plasmid was cured to create a strain that is unable to grow on arabinose without a suitable transporter (strains BFY518 and BFY590). *E. coli* was grown in LB medium or on LB agar plates supplemented with ampicillin at 100 μg/ml. Overexpression plasmids were constructed by cloning the gene of interest downstream of the *S. cerevisiae PGK1* promoter of 2 μ-based *HIS3* or *URA3* yeast selection vectors. For *K. marxianus* genomic library construction details, see Results section.

Differential display

Cells were grown in YNB medium with either arabinose or xylose as the carbon source. When cultures reached an OD_{600} of approximately 2.0, 15 ml cells were added to 25 ml crushed ice in a 25 ml conical tube and pelleted at 4 °C at 4500 rpm for 5 min. The cells were resuspended in 1 ml ice-cold DEPC-treated water and moved to a 1.5 ml Eppendorf tube. The cells were again spun at maximum speed in a microcentrifuge and all supernatant was removed. The cell pellets were frozen on dry ice and stored at −80 °C. Cell pellets were thawed on ice and immediately resuspended in 400 μl TES solution (10 mM Tris–HCl, 5 mM EDTA, 0.5% w/v SDS, pH 7.5). An additional 400 μl acidic, non-

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buffered, water-saturated phenol was added and the cells were vigorously vortexed for 10 s. The tubes were then incubated for 60 min at 65 °C with occasional, brief vortexing. Following this incubation, the tubes were spun at 4 °C at 15 000 × *g* for 5 min and the aqueous supernatant was transferred to a fresh tube, where 400 µl acidified phenol was added, vortexed and spun as before. The aqueous supernatant was transferred to a fresh tube and 400 µl chloroform was added. Vortexing and centrifugation were repeated. The aqueous portion was transferred to a fresh tube and 30 µl 3 M sodium acetate, pH 5.3, was added, followed by 857 µl ice-cold 100% ethanol. The tube was incubated at 20 °C for 30 min then centrifuged at 4 °C for 5 min at top speed. The supernatant was removed and the RNA pellet washed with 1 ml 70% ethanol, followed by centrifugation at 4 °C for 5 min. The pellet was then air-dried at room temperature for 1 h and suspended in 50 µl DEPC-treated water and frozen. Isolated RNA (60 µg) was sent to GenHunter (www.genhunter.com) for differential display analysis.

Growth curves

Growth curves on arabinose and xylose were done in shake flasks as follows. Overnight cultures were grown in YNB lacking tryptophan and uracil. Cells were harvested, washed and resuspended in sterile water. The cells were then inoculated into shake flasks containing either 20 g/l arabinose or xylose YNB medium. The flasks were incubated at 30 °C and 250 rpm. Growth curves in the BioScreenC (GrowthCurvesUSA, NJ, USA) were performed as follows. Strains were grown overnight in YNB lacking uracil and containing 2% maltose. The cells were harvested, washed, resuspended in sterile water and inoculated into YNB medium containing galactose, glucose, maltose or no sugar. Quadruplicates of each culture were aliquoted (300 µl) into wells in the honeycomb BioScreenC plate and wide-band OD (420–580 nm) was recorded every 15 min. Growth temperature was 30 °C. Shaking of the BioscreenC plate was performed for 10 s prior to each reading.

HPLC

Utilization of arabinose was determined by measuring the sugar remaining in the medium after various periods of growth, by the analysis of filtered media using high-performance liquid chromatography (HPLC) on a Hewlett-Packard (HP) 1090 instrument using a Bio-Rad HPX-87H hydrogen ion resin column and an HP 1047A external refractive index detector. The mobile phase, 0.001 N H_2SO_4, was run at 55 °C at a flow rate of 0.6 ml/min.

Transformation

Electrotransformation of *E. coli* Dh5α was performed as described (Invitrogen 11319–019) and plated on LB plates containing 100 µg/ml ampicillin. Transformation of *S. cerevisiae* was performed using DMSO-enhanced lithium acetate (Hill *et al.,* 1991), with the following modifications. The cells were initially washed in water; 600 µl PEG4000 solution was added and, just prior to heat shocking, 70 µl DMSO was added. The cells were heat-shocked for 15 min at 42 °C and the last wash step was eliminated. The cells were resuspended in 10 mM TES and plated on appropriate selective plates.

DNA manipulations

Yeast DNA was isolated using the Easy DNA Kit, as described (Invitrogen, K1800-01). DNA manipulations and library construction were done as described (Sambrook *et al.,* 1989). Plasmids were cured from yeast by growing the strain in rich, non-selective medium (YPD) overnight, followed by plating on non-selective medium. Isolated colonies were replica-plated to screen for the loss of selective markers. Plasmid rescue was performed by transforming isolated yeast DNA into *Escherichia coli*, followed by isolation and characterization. *E. coli* plasmid DNA was isolated using a plasmid spin mini-prep kit (Qiagen, 27106). Chromosomal PCR-based walking was performed, using the Universal GenomeWalker Kit as described (BD Biosciences, K1807-1).

Transport assays

Cells were grown in YNB medium supplemented with 20 g/l arabinose and lacking tryptophan and uracil, for plasmid maintenance. Cells were collected in mid-growth and washed twice before suspension in water at 30 mg/ml. Uptake of L-(1-^{14}C) arabinose (54 mCi/mM; Moravek Biochemicals Inc.) or D-(1-^{14}C)galactose (57 mCi/mM; Amersham

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Biosciences) was measured in triplicate, as previously described (Stambuk *et al.,* 2003). The radiolabelled L-arabinose was a custom-synthesized batch, due to contamination in the radiolabelled L-arabinose sourced from American Radiolabelled Chemicals, as described previously (Knoshaug *et al.,* 2009). Assays were performed in 30 s to maintain initial rates after appropriate experiments ensured that uptake was linear for at least 1 min. Transport activity is described as nM labelled sugar transported/mg cell dry weight/min. Inhibition and competition assays were performed as previously described (Stambuk *et al.,* 2003). Xylose transport was performed as previously described (Lucero *et al.,* 1997). Cells were grown in YNB medium lacking uracil and supplemented with 20 g/l maltose to middle logarithmic growth phase, harvested by centrifugation (10 min, 6000 rpm, 0 °C), washed with ice-cold water and then with ice-cold 0.1 M tartrate/Tris, pH 4.2, and finally suspended in the same buffer to 200 mg fresh yeast/ml. Zero-trans D-(U-^{14}C)xylose (85 mCi/mM; Amersham Biosciences) uptake rates were immediately determined.

Results

Isolation of the *KmAXT1* gene

A *K. marxianus* genomic library was constructed in yeast vector pBFY12, which contains the 2 μ origin, a *URA3* selection cassette and a *Bam*HI site located between the *PGK1* promoter and *GAL10* terminators from *S. cerevisiae*. After partial digestion of 200 μg genomic DNA with *Sau*3AI restriction enzyme, fragments of 2–8 kb length were gel-isolated and ligated into the *Bam*HI site of pBFY012. This ligation reaction was then transformed into *E. coli* and plated for recovery. Plate counts produced ~3000 cfu/10 μl transformed cells and the plasmid DNA from 24 colonies was screened for the presence of an insert, revealing that 22 of 24 transformants had an insert in the range 1–10 kb, giving an average insert size of 3.2 kb. The transformed cells were scraped from the plates, DNA recovered, and 5 μl was transformed into competent BFY518 cells. The strain BFY518 was previously cured of the *GAL2* overexpression plasmid, eliminating its ability to form colonies on agar plates containing arabinose as the sole carbon source. Colony formation could then be restored by complementation with a heterologous arabinose transporter from the library. The total number of transformants screened for growth on arabinose was estimated by plating a small sample of the transformation mixture on minimal glucose medium. The remainder of the transformation mix was plated onto minimal medium containing 2% arabinose for selection. The total number of cells plated for selection was approximately 280 000, representing about eight-fold coverage of the 10.7 mb *K. marxianus* genome (Dujon *et al.,* 2004). Many small colonies appeared on these plates after 3 days and were replica-plated to fresh arabinose minimal medium. Two colonies grew on the replica plates. Plasmid DNA was rescued and retransformed into BFY518, which allowed growth once again on arabinose, confirming that the *K. marxianus* genomic insert carried on these plasmids was responsible for growth. Restriction digestion analysis of the plasmids recovered from the transformants growing on arabinose showed that both transformants harboured a plasmid, now designated pBFY171, with the same insert of approximately 8.8 kb in size.

Isolation of the *PgAXT1* gene

Differential display was utilized for the isolation of an arabinose transporter from *P. guilliermondii*, since direct complementation using a genomic library from this species would not work, as this species is known to use a non-standard genetic code (Tuite and Santos, 1996; Sugita and Nakase, 1999). The induction of arabinose transport in wild-type *P. guilliermondii* was first examined. Only cells grown on arabinose were able to transport arabinose (Figure 1). Additionally, xylose transport was about double in cells grown in arabinose compared to cells grown in xylose. Galactose was transported at the same rate, independent of growth substrate. Transport competition between arabinose and xylose was also examined. Uptake of labelled arabinose was reduced by 96% when 100× unlabelled xylose was included in the transport assay, whereas uptake of labelled xylose was only reduced by 16% when 100× unlabelled arabinose was included in the assay (Figure 2). These data suggest that in *P. guilliermondii*, growth on arabinose induces expression of a specific transport system capable of transporting arabinose and xylose. This system preferentially transports xylose

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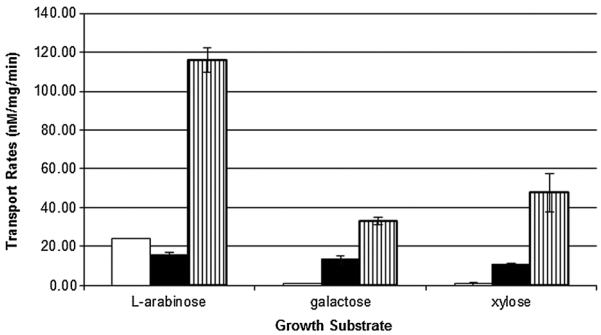

Figure 1. Induction of arabinose transport in *P. guilliermondii*. Cells grown in minimal medium containing 2% arabinose, galactose or xylose were assayed for uptake of 13 mM labelled arabinose (white bars), galactose (black bars) or xylose (vertical stripes)

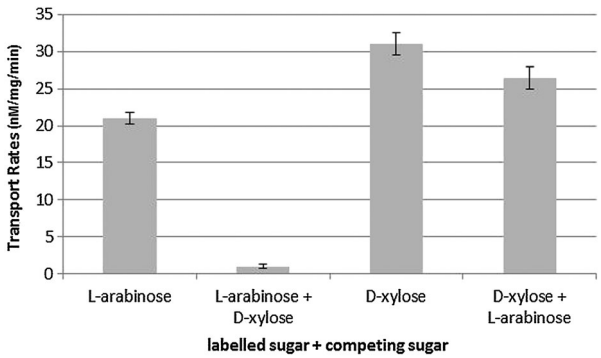

Figure 2. Transport competition analysis in *P. guilliermondii* grown in minimal arabinose medium. Labelled sugar uptake was assayed at 1.2 mM, with or without the addition of 120 mM unlabelled competing sugar, as indicated

at the expense of arabinose if both sugars are present and has a higher transport velocity for xylose than the transport system induced when grown solely on xylose. Thus, transport activity for arabinose is not induced when grown on xylose, validating our choice of sugars for growth and differential display. Differential display cDNA libraries from *P. guilliermondii* were prepared from cultures grown in a medium with either xylose or arabinose as the sole carbon source; 116 transcripts that were greatly enhanced in the cultures grown on arabinose, as compared to the analogous cultures grown on xylose, were identified and annotated using the Genbank sequence database (data not shown). Of the 116 differentially regulated transcripts, 49 did not return significant sequence matches, while four transcript sequences showed similarity to sugar transporter sequences of other fungi [arabinose differential display (ADD) transcripts 011, 023, 096 and 097]. From the limited transcript sequence data, we employed genome walking to recover the upstream and downstream full-length coding regions of each gene. During this process, some genes were more readily isolated than others and we were thus only able to realize full-length sequences for ADD023 and ADD096.

Sequence analysis of the functional library and differential display transporters

Sequencing results of the complementing plasmid from the *K. marxinaus* library contained an insert of 8838 kb, having two ORFs on the 5′ end of the insert (Figure 3). Both of these ORFs showed strong homology to yeast sugar transporters. One transporter ORF was interrupted by a fragment of an unrelated ORF, suggesting that recombination of fragments during ligation into the vector occurred in library construction. This was confirmed by PCR walking experiments performed on *K. marxianus* genomic DNA. Walking was performed in a 5′ direction relative to the ORF and additional transporter sequence, including the start codon, was recovered, rather than the

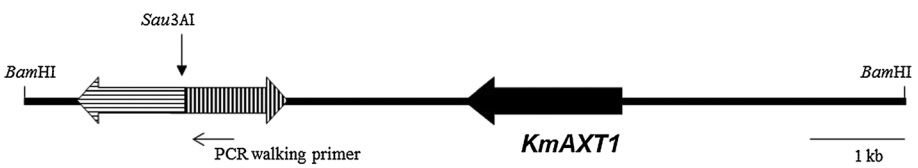

Figure 3. Library insert from genomic *K. marxianus* DNA complements adapted *S. cerevisiae*, allowing growth on arabinose. Cloning into the expression vector is at the indicated *Bam*HI restriction sites. The black block arrow is the arabinose transporter ORF responsible for complementation (*KmAXT1*). The block arrow with vertical stripes is the interrupted transporter ORF. The block arrow with the horizontal stripes is an unrelated ORF ligated in place gratuitously during library construction at the indicated *Sau*3AI restriction site. The primer used for PCR-based genomic walking in *K. marxianus* is shown

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sequence from the unrelated ORF present in the plasmid (data not shown). Since this transporter ORF was interrupted and missing a substantial portion of the 5′ region, including a start codon, we reasoned that this ORF could not be the complementing ORF. Indeed, subsequent cloning and expression of the full-length ORF of this transporter in an arabinose-adapted *S. cerevisiae* strain did not restore growth on arabinose, in contrast to the other ORF present on this genomic library fragment. The uninterrupted transporter ORF was recovered twice more in a subsequent library screening (data not shown). This ORF was 1668 bp in length (Accession number for *KmAXT1*: GZ791039) and shared homology with high-affinity glucose transporters.

The two full-length sugar transporter sequences identified by differential display (ADD023 and ADD096) were analysed for CTG codons. For successful expression in *S. cerevisiae*, these codons would have to be changed to a standard serine codon, since CTG codes for serine instead of leucine in *P. guilliermondi.* In the ADD023 sequence there was one CTG codon, whereas in the ADD096 sequence none of the 34 leucine codons was CTG. Sequence analysis results of the *ADD096d* gene showed an ORF 1617 bp in length (Accession No. for *PgAXT1*, GZ791040).

Transmembrane region prediction (http://www.ch.embnet.org/software/TMPRED_form.html) (Hofmann and Stoffel, 1993) was performed on both transporter protein sequences and 12 membrane-spanning regions, with a larger intercellular loop between regions 6 and 7 was found, typical of *GAL2* and other yeast sugar transporters having 10–12 transmembrane regions (Kruckeberg, 1996; Weierstall *et al.,* 1999; Day *et al.,* 2002; Alves-Araujo *et al.,* 2004; Pina *et al.,* 2004). The two transporters were phylogenetically compared to existing transporters known to transport either arabinose or xylose (Figure 4). Transporters that strongly transport either xylose (DhXylhp, SsXut1p, SsXut3p) or arabinose (AmLat1p, AmLat2p, KmAxt1p, PgAxt1p, and SsAraTp) grouped together, whereas those that transport either pentose but strongly prefer glucose also grouped together. A substrate recognition motif in the first membrane-spanning region has been described,

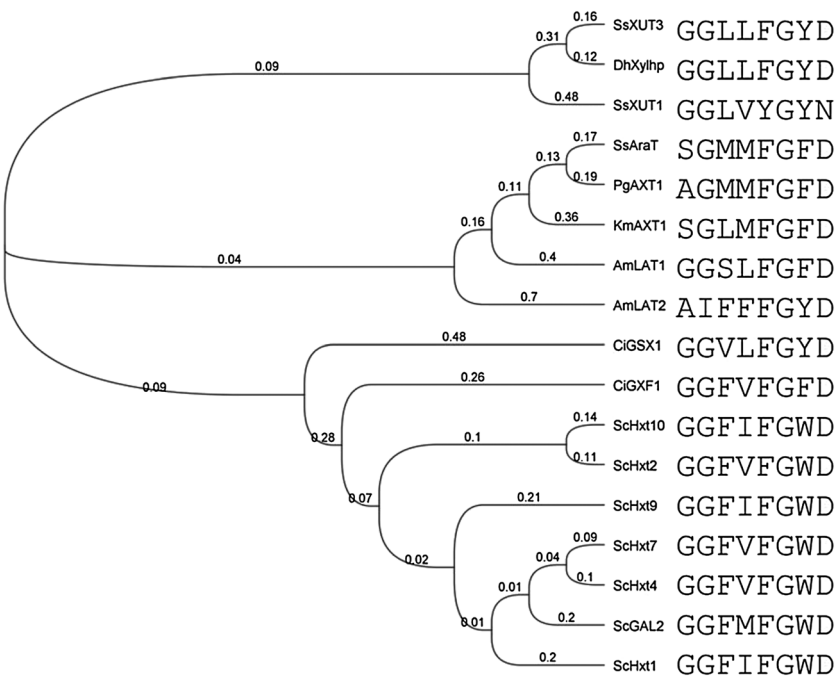

Figure 4. Phylogenetic analysis of KmAXT1 and PgAXT1 transporter proteins. The phylogenetic tree analysis and multiple protein alignment was assembled using Genious 7.1.7 Tree Builder and multiple alignment algorithm, using a global alignment with free end gaps and the Blosum62 cost matrix. Ss, *Scheffersomyces stipitis*; Dh, *Debaryomyces hansenii*; Pg, *Pichia guilliermondii*; Km, *Kluyveromyces marxianus*; Am, *Ambrosiomyces monospora*; Ci, *Candida intermedia*; Sc, *Saccharomyces cerevisiae*

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G–G/F–X–X–X–G (Young *et al.,* 2014). Alignment of these proteins shows clear differences in this motif group together based on sugar transport preferences of the transporters (Figure 4). Xylose transporters favour G–G–L–X–X–G–Y–D/N, whereas arabinose transporters favour the sequence X–G–X–X–F–G–F–D. The transporter group strongly favouring glucose have the sequence G–G–F–X–F–G–W–D. Interestingly, Gal2p contains a methionine in the fourth position similar to three of the arabinose-preferring transporters.

Gal2p, KmAxt1p and PgAxt1p support growth on arabinose and xylose

The protein-coding sequences of *KmAXT1* and *PgAXT1* were isolated via PCR from genomic DNA of *K. marxianus* and *P. guilliermondii*, respectively, and cloned for overexpression using the *PGK1* promoter of *S. cerevisiae*. These constructs were transformed into an arabinose-adapted strain from which the endogenous copy of *GAL2* had been entirely replaced with a *HIS3* selection marker (BFY590). Growth curve results clearly showed that KmAxt1p (BFY598), PgAxt1p (BFY605) or Gal2p (BFY597) are required to rescue growth on arabinose when compared to the empty vector control (BFY596), confirming that the genes cloned express transporters necessary for growth on arabinose in the absence of the Gal2 permease (Figure 5a). These data also suggest that none of the native glucose transporters transport enough arabinose to support rapid growth, or that these transporters are not expressed when grown on arabinose. These vectors were also transformed into the H2219 host (Saloheimo *et al.,* 2007) and shown to allow growth on xylose (Figure 5b). The long delay prior to growth is due to unknown adaptations occurring in the host and the small increase in density in the empty vector control strain has been noted previously (Saloheimo *et al.,* 2007). The vectors carrying *KmAXT1*, *PgAXT1*, and *GAL2* were also transformed into BFY85, a strain of *S. cerevisiae* that has been deleted for *HXT1–HXT7*, *GAL2* and *SUC2* transporters and is unable to grow on glucose, galactose or sucrose in the absence of a suitable transporter. Comparing growth of these strains, it is clear that neither KmAxt1p (BFY585) nor PgAxt1p (BFY690) transport either glucose or galactose in sufficient quantities to support growth, while the same strain overexpressing Gal2p (BFY584) grew readily on both glucose and galactose (Figure 6). The empty vector strain (BFY583) grew only on maltose.

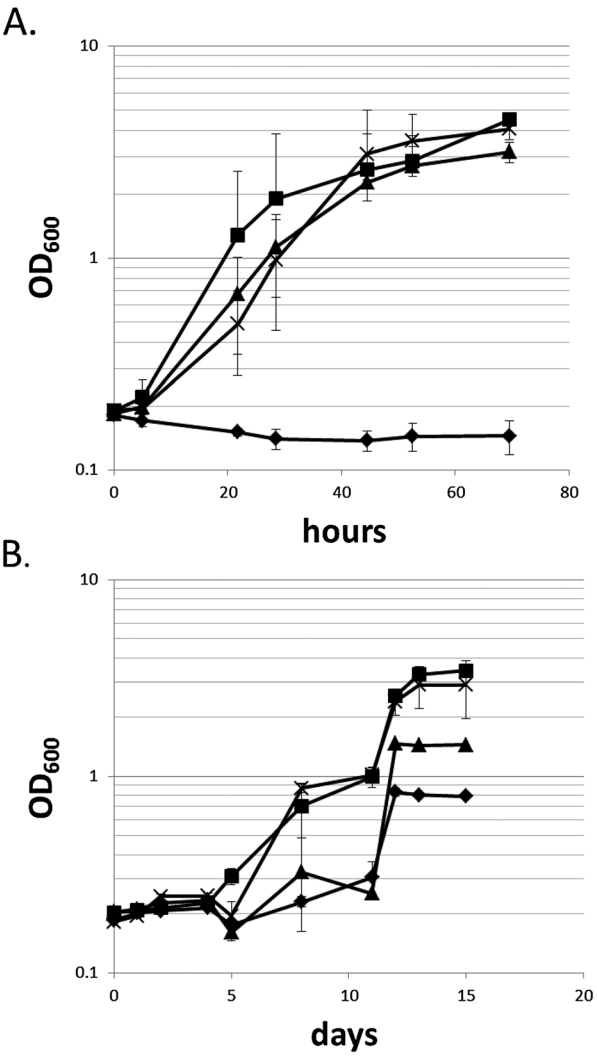

Figure 5. Growth of *S. cerevisiae* expressing GAL2 (square), KmAXT1 (triangle), PgAXT1 (X) or empty vector (diamond) on minimal medium with: (A) 2% arabinose; (B) 2% xylose and 0.06% maltose as a carbon source

Arabinose and xylose transport kinetics of Gal2p, KmAxt1p and PgAxt1p expressed in *S. cerevisiae*

In order to minimize the contribution to arabinose transport of native transporters, we deleted the native *GAL2* gene to create a strain that is adapted for growth on arabinose but unable to transport arabinose (BFY590). The transporter expression

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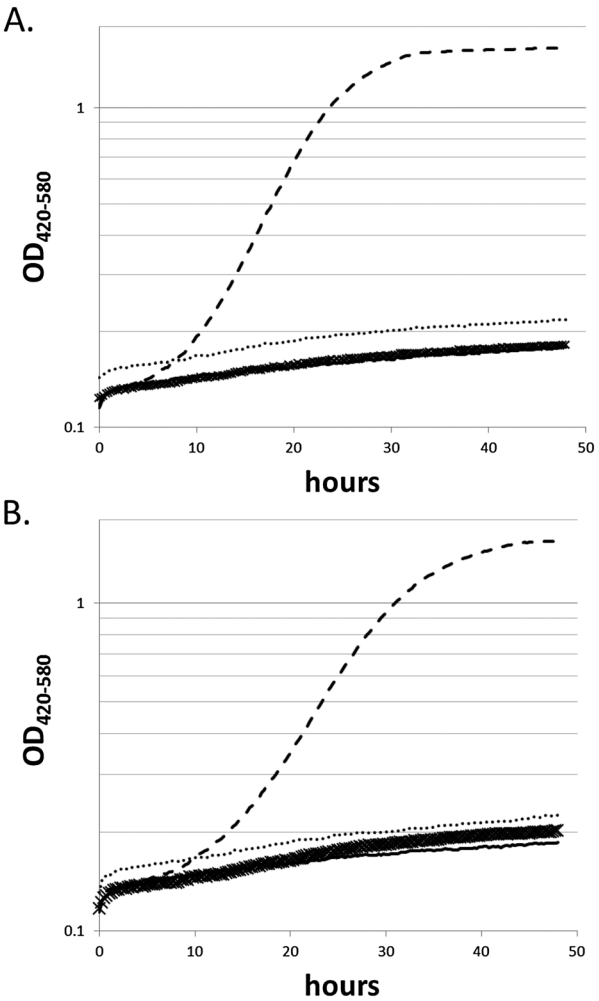

Figure 6. Growth curves generated in the BioscreenC for *S. cerevisiae* strains grown on A. glucose or B. galactose. BFY583 (empty vector) dotted line, BFY584 (GAL2) dashed line, BFY585 (KmAXT1) X line, and BFY690 (PgAXT1) solid line.

plasmids were then established in this strain for the determination of arabinose transport kinetics. The KmAxt1p transporter showed a low-affinity transporter having K_m 263 mM and V_{max} 57 nM/mg/min (Table 2). This is in contrast to the high-affinity active transport activity induced in the wild-type *K. marxianus* when grown on 2% arabinose (Knoshaug *et al.,* 2009). These results suggest there are at least two transporters in *K. marxianus* that can transport arabinose but that only the high-affinity activity is induced in the wild-type when grown on 2% arabinose. Inhibition experiments showed that when KmAxt1p is expressed in *S. cerevisiae* it is not significantly inhibited by the protonophores (NaN_3, DNP and CCP) and H^+ adenosine triphosphatase (ATPase) inhibitors (DESB and DCCD) (Table 3), in contrast to the wild-type activity in *K. marxianus* (Knoshaug *et al.,* 2009), suggesting that KmAxt1p is a facilitated diffusion permease similar to the Gal2 permease. Competition experiments showed that arabinose transport by KmAxt1, when expressed in *S. cerevisiae*, is inhibited by glucose, galactose and xylose (Table 3).

The arabinose transport characteristics of the PgAxt1p transporter expressed in *S. cerevisiae* showed the same arabinose transport characteristics as the wild-type *P. guilliermondii* (Knoshaug *et al.,* 2009). The PgAxt1p transporter, when expressed in *S. cerevisiae* has K_m 0.13 mM and V_{max} 18 nM/mg/min (Table 2). Inhibition experiments show significant inhibition of transport by protonophores (NaN_3, DNP and CCP) and H^+ adenosine triphosphatase (ATPase) inhibitors (DESB and DCCD) similar to inhibition in wild-type *P. guilliermondii* (Knoshaug *et al.,* 2009) (Table 3). Competition experiments show that arabinose uptake by the PgAxt1 transporter is inhibited by glucose and galactose and completely blocked by xylose (Table 3). Xylose transport is nearly 90% of normal levels when competed against arabinose. These two results suggest that the PgAxt1 transporter is thus primarily a xylose transporter.

Transport kinetics of *S. cerevisiae* BFY597 overexpressing the Gal2 permease showed K_m

Table 2. Substrate affinity (K_m) and rate (V_{max}) of L-arabinose and D-xylose transport in *Saccharomyces cerevisiae* expressing *GAL2*, Km*AXT1* or Pg*AXT1*

	L-Arabinose transport		D-Xylose transport	
Transporter	**K_m (mM)**	**V_{max} (nM/min/mg)**	**K_m (mM)**	**V_{max} (nM/min/mg)**
GAL2	371 ± 19	341 ± 7	ND	ND
KmAXT1	263 ± 57	57 ± 6	27 ± 3	3.8 ± 0.02
PgAXT1	0.13 ± 0.04	18 ± 0.8	65 ± 8	8.7 ± 0.3

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Table 3. Effect of inhibitors or competing sugars on the rate of L-arabinose or D-xylose transport in *Saccharomyces cerevisiae* expressing *GAL2*, Km*AXT1* or Pg*AXT1*

		Relative L-arabinose transport (%)		
Inhibitor or competing sugar	**Concentration (mM)**	**Gal2p**	**KmAxt1p**	**PgAxt1p**
None	–	100[1]	100[3]	100[5]
NaN_3	10	66	11	16
CCCP	5	46	61	2
DCCD	5	69	55	36
DNP	5	72	75	4
DESB	5	81	100	1
None	–	100[2]	100[4]	100[6]
Glucose	120	10	17	17
Galactose	120	3	23	20
D-Xylose	120	25	25	0
			Relative D-xylose transport	
None	–	–	100[7]	100[8]
L-Arabinose	250	–	82	88

[1]Uptake rate was 66.0 nM/mg/min determined with 118 mM labelled L-arabinose.
[2]Uptake rate was 18.9 nM/mg/min determined with 30 mM labelled L-arabinose.
[3]Uptake rate was 7.7 nM/mg/min determined with 118 mM labelled L-arabinose.
[4]Uptake rate was 3.6 nM/mg/min determined with 30 mM labelled L-arabinose.
[5]Uptake rate was 11.2 nM/mg/min determined with 0.33 mM labelled L-arabinose.
[6]Uptake rate was 14.2 nM/mg/min determined with 1.2 mM labelled L-arabinose.
[7]Uptake rate was 2.8 nM/mg/min determined with 50 mM labelled D-xylose.
[8]Uptake rate was 3.2 nM/mg/min determined with 50 mM labelled D-xylose.

371 mM and V_{max} 341 nM/mg/min for arabinose transport (Table 2). Inhibition assays showed a reduction, but not a complete inhibition, of transport suggestive of facilitated diffusion transport, as previously reported (Kou *et al.,* 1970) (Table 3). Competition studies showed that glucose, galactose and xylose significantly reduced arabinose transport, indicating that these sugars are transported preferentially over arabinose (Table 3). The kinetics of galactose transport were also measured in this strain and indicated that Gal2p has K_m 25 mM and V_{max} 76 nM/mg/min for galactose transport, demonstrating a higher affinity for galactose that would out-compete arabinose for transport.

The *KmAXT1* and *PgAXT1* transporter expression plasmids were established in the H2219 host in order to characterize xylose transport free from additional transport due to the Hxt or Gal2 transporters that are known to transport xylose. Xylose transport kinetics showed K_m 27 and 65 mM and V_{max} 3.8 and 8.7 nM/mg/min for KmAxt1p and PgAxt1p, respectively (Table 2). Competition studies showed that xylose is preferentially transported over arabinose, as a 5× concentration of arabinose only reduced xylose transport by 10–20% (Table 3).

Discussion

Functional complementation is an effective method for cloning novel genes and has been used to isolate glucose transporters from *P. stipitis*, a maltose transporter from *Torulaspora delbrueckii*, a fructose transporter from *Zygosaccharomyces bailii*, an arabinose transporter from *Scheffersomyces stipitis* (Weierstall *et al.,* 1999; Alves-Araujo *et al.,* 2004; Pina *et al.,* 2004; Subtil and Boles, 2011) and xylose transporters from *Candida intermedia*, *Neurospora crassa* and *Pichia stipitis* (Leandro *et al.,* 2006; Du *et al.,* 2010). High-capacity arabinose transport at low concentrations has previously been described in the arabinose-utilizing yeasts *K. marxianus* and *P. guilliermondii* (Knoshaug *et al.,* 2009). We successfully screened a genomic library and isolated an arabinose transporter from *K. marxianus* by complementing *S. cerevisiae* that had been adapted for growth on arabinose but lacked sufficient arabinose transport for effective growth. The transport profile of wild-type *K. marxianus* leads one to expect that a single, high-affinity, active transporter would be isolated by complementation, yet we isolated a transporter having low-affinity, facilitated diffusion activity that was not observed in the

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wild-type transport profile of *K. marxianus* when grown on 2% arabinose. These fundamental differences in transport characteristics and inhibition profiles between the cloned KmAxt1 transporter and wild-type *K. marxianus* transport activity make it highly unlikely that they are due to altered glycosylation patterns or poor expression in *S. cerevisiae*. Using differential display, we successfully isolated a high-affinity arabinose transporter induced by growth on arabinose in wild-type *P. guilliermondii*. The PgAxt1 (ADD096) transporter has very similar transport characteristics to the single, high-affinity arabinose transporter we previously characterized (Knoshaug *et al.,* 2009). Also of note, because no CTG codons were present in the *PgAXT1* open reading frame, screening an expression library of *P. guilliermondii* probably would have been successful for the isolation of *PgAXT1*.

Comparing the transport activities of KmAxt1p or PgAxt1p to Gal2p shows that Gal2p is superior in transporting arabinose and has a higher capacity at any given concentration. This is manifested in growth curves showing that cells that are expressing *KmAXT1* or *PgAXT1* grow at a slower rate, and cells expressing *KmAXT1* do not reach as high a final density. The results are similar for KmAxt1p when transporting xylose. PgAxt1p, however, supports as robust growth as Gal2p on xylose. It is puzzling that KmAxt1p seems to be a relatively poor transporter of arabinose or xylose compared to other transporters, yet also does not effectively transport glucose or galactose, as evidenced by lack of growth in BFY85 on glucose or galactose. Clearly our cloning strategy was successful, and the discovery of a novel arabinose transporter that was not predicted by the characterization of wild-type transport of arabinose-grown cells highlights the point that there are a suite of transporters present in arabinose-utilizing yeasts capable of transport, but that are inactive or not expressed during growth on a particular substrate. This also suggests that the transporter we isolated is not the major arabinose transporter used by *K. marxianus*, but perhaps a transporter of some other sugar that also recognizes and transports arabinose and xylose. In addition, some transporters, when expressed in *S. cerevisiae*, do not behave as they do in their native organism (Young *et al.,* 2011; Ferreira *et al.,* 2013).

Investigations of the best known yeast arabinose transporter demonstrated thus far, Gal2p, have been limited. Previous reports showed that Gal2p is a facilitated diffusion permease only transporting arabinose as long as the external concentration is higher than the internal cellular concentration, and showed inducible transport of arabinose at 167 mM when cells are grown on galactose; however, the quantity of cells used was not specified, so a direct comparison could not be made concerning K_m and V_{max} (Cirillo, 1968; Kou *et al.,* 1970). More recently, Gal2p has been confirmed as a facilitated diffusion permease by counter-transport studies (Maier *et al.,* 2002) and our study showing that arabinose transport was not significantly affected by common protonophores (NaN_3, DNP and CCP) or H+-adenosine triphosphatase (ATPase) inhibitors (DESB and DCCD). Another measure of arabinose transport by Gal2p showed a velocity of 0.32 nM/mg/min at 10 mM (Becker and Boles, 2003). Surprisingly, our transport assay gave a value 28 times greater (8.9 nM/mg/min) for arabinose transport at a similar concentration (11.8 mM). Both studies were done using strains that overexpressed Gal2p, but different promoters were used. In our study, *GAL2* overexpression was driven by the *TDH3* promoter, whereas the *ADH1* promoter was used in the earlier study. Whether this makes any difference in transporter expression levels is not clear, but two previous reports indicate that the *TDH3* promoter gave 10 times more activity of β-galactosidase than the *ADH1* promoter from a 2 μ-based plasmid, and the *TDH3* promoter delivered the highest activity among the seven promoters assayed (Mumberg *et al.,* 1995; Nacken *et al.,* 1996). A difference in expression levels could generate higher velocities at any given concentration if more individual transporters were present. Additionally, Hxt9p and Hxt10p have been shown to transport arabinose sufficiently for growth, but Gal2p remains a better arabinose transporter (Subtil and Boles, 2011).

Our results show that the Gal2 permease has a much lower affinity for arabinose than for galactose. This pattern has also been observed for the *HXT* transporters, in that they also possess a lower affinity for sugars other than their primarily transported sugar, glucose (Bisson and Fraenkel, 1984; Wendell and Bisson, 1994). In contrast, Gal2p has been reported to have a higher affinity for glucose, 1–2 mM (Reifenberger *et al.,* 1997), than the 25 mM we found for galactose. The much higher affinity for glucose explains why arabinose transport is blocked by the presence of glucose in

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the transport assays. Also, due to the differing affinities, galactose transport is probably likewise affected by glucose at the transporter as well as the regulatory level, as previously described (Tschopp *et al.,* 1986; Huibregtse *et al.,* 1993). The Gal2 permease has also been shown to effectively transport xylose to support growth (Hamacher *et al.,* 2002; Sedlak and Ho, 2004; Saloheimo *et al.,* 2007). Thus, with a single facilitated diffusion transporter, the major hexose and both major pentose sugars present in biomass hydrolysates can be transported into the cell without the use of ATP for transport. However, arabinose transport by Gal2p is out-competed by glucose, galactose and xylose and would presumably occur only after these sugars have been utilized, increasing the length of the fermentation when arabinose use is desired for additional product formation.

Pentose transport needs to be improved for efficient pentose utilization and economic viability of biofuels production. However, a recent survey of xylose transporters showed that, although they could transport xylose, glucose was the preferred substrate (Young *et al.,* 2011), highlighting the problem with glucose and co-fermentation. Some progress is being made. Mutagenesis achieved a strain that co-fermented xylose and glucose where xylose was transported from the start, although slowly until glucose was exhausted. Thus, it is likely there were mutational changes residing within a transporter; however, they did not sequence the transporters (Demeke *et al.,* 2013). Direct engineering of transport proteins is also showing results. Glucose effectively binds and thus blocks transport of pentose sugars by Gal2p. The glucose and galactose recognition sites have been identified and altered to improve the transport of xylose in the presence of glucose (Kasahara and Kasahara, 2000; Farwick *et al.,* 2014), and additional substrate recognition engineering to block glucose inhibition of xylose transport has also been done (Young *et al.,* 2014). The expanded set of preferred amino acids spanning the recognition sites in the first transmembrane region of the transporters, as shown in Figure 4, may provide useful targets for transporter substrate engineering.

It has been previously postulated that for efficient and complete conversion of biomass pentose sugars, both low-affinity, high-throughput and high-affinity active transporters for arabinose and xylose will be needed to efficiently ferment all the available pentose sugars in any given substrate (Lee *et al.,* 2002; Subtil and Boles, 2011). Indeed, broad substrate range transporters have been shown to confer higher growth rates than specific transporters (Young *et al.,* 2011). Here we demonstrate that we have isolated and characterized two transporters that transport both xylose and arabinose. Many of the previously described xylose or arabinose transporters have only been characterized for transport of either xylose or arabinose and, from our work, it appears very likely that these other described transporters may in fact transport both pentose sugars. Thus, in a scenario utilizing Gal2p for the bulk of the pentose sugar transport, only one other high-affinity transporter is probably needed for complete pentose usage.

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