The *Penicillium chrysogenum* transporter *PcAraT* enables high-affinity, glucose-insensitive \(\text{l}-\text{arabinose}\) transport in *Saccharomyces cerevisiae*

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**Abstract**

**Background:** \(\text{l}-\text{Arabinose}\) occurs at economically relevant levels in lignocellulosic hydrolysates. Its low-affinity uptake via the *Saccharomyces cerevisiae* Gal2 galactose transporter is inhibited by \(\text{d}-\text{glucose}\). Especially at low concentrations of \(\text{l}-\text{arabinose}\), uptake is an important rate-controlling step in the complete conversion of these feedstocks by engineered pentose-metabolizing *S. cerevisiae* strains.

**Results:** Chemostat-based transcriptome analysis yielded 16 putative sugar transporter genes in the filamentous fungus *Penicillium chrysogenum* whose transcript levels were at least threefold higher in \(\text{l}-\text{arabinose}\)-limited cultures than in \(\text{d}-\text{glucose}\)-limited and ethanol-limited cultures. Of five genes, that encoded putative transport proteins and showed an over 30-fold higher transcript level in \(\text{l}-\text{arabinose}\)-grown cultures compared to \(\text{d}-\text{glucose}\)-grown cultures, only one (Pc20g01790) restored growth on \(\text{l}-\text{arabinose}\) upon expression in an engineered \(\text{l}-\text{arabinose}\)-fermenting *S. cerevisiae* strain in which the endogenous \(\text{l}-\text{arabinose}\) transporter, *GAL2*, had been deleted. Sugar transport assays indicated that this fungal transporter, designated as *PcAraT*, is a high-affinity (\(K_m = 0.13\) mM), high-specificity \(\text{l}-\text{arabinose}\)-proton symporter that does not transport \(\text{d}-\text{xylose}\) or \(\text{d}-\text{glucose}\). An \(\text{l}-\text{arabinose}\)-metabolizing *S. cerevisiae* strain in which *GAL2* was replaced by *PcaraT* showed 450-fold lower residual substrate concentrations in \(\text{l}-\text{arabinose}\)-limited chemostat cultures than a congenic strain in which \(\text{l}-\text{arabinose}\) import depended on Gal2 (4.2 \(\times\) \(10^{-3}\) and 1.8 g L\(^{-1}\), respectively). Inhibition of \(\text{l}-\text{arabinose}\) transport by the most abundant sugars in hydrolysates, \(\text{d}-\text{glucose}\) and \(\text{d}-\text{xylose}\) was far less pronounced than observed with Gal2. Expression of *PcAraT* in a hexose-phosphorylation-deficient, \(\text{l}-\text{arabinose}\)-metabolizing *S. cerevisiae* strain enabled growth in media supplemented with both 20 g L\(^{-1}\) \(\text{l}-\text{arabinose}\) and 20 g L\(^{-1}\) \(\text{d}-\text{glucose}\), which completely inhibited growth of a congenic strain in the same condition that depended on \(\text{l}-\text{arabinose}\) transport via Gal2.

**Conclusion:** Its high affinity and specificity for \(\text{l}-\text{arabinose}\), combined with limited sensitivity to inhibition by \(\text{d}-\text{glucose}\) and \(\text{d}-\text{xylose}\), make *PcAraT* a valuable transporter for application in metabolic engineering strategies aimed at engineering *S. cerevisiae* strains for efficient conversion of lignocellulosic hydrolysates.

**Keywords:** *Penicillium*, Transcriptome, Sugar transport, Proton symport, \(\text{l}-\text{Arabinose}\) transporter, Second-generation bioethanol, Yeast, Metabolic engineering
Background

At an annual production of 100 Mton [1], bioethanol produced by the yeast *Saccharomyces cerevisiae* is by volume the largest fermentation product in industrial biotechnology. Cane sugar and corn starch, which are still the predominant feedstocks for bioethanol production, almost exclusively yield sucrose and D-glucose as fermentable sugars. Alternative lignocellulosic feedstocks, derived from agricultural residues or energy crops, contain cellulose, hemicellulose, and in some cases, pectin [2]. The pentoses D-xylose and L-arabinose typically represent 10–25 and 2–3%, respectively, of the monomeric sugars in lignocellulosic hydrolysates [3]. Some industrially relevant hydrolases, however, contain higher L-arabinose concentrations. For instance, in hydrolysates of corn fibre and sugar beet pulp, L-arabinose represents 16 and 26% of the total sugar content, respectively [4, 5].

Whilst pentose sugars are not natural substrates of *S. cerevisiae*, their efficient conversion to ethanol and, ultimately, other bulk products, is essential to ensure economically viable processes [6]. Extensive metabolic and evolutionary engineering has been applied to enable efficient xylose fermentation, based on expression of either a heterologous xylose reductase and xylitol dehydrogenase, or a heterologous xylose isomerase (reviewed by [7] and [8]). Construction of yeast strains capable of L-arabinose fermentation involved functional expression of bacterial genes encoding L-arabinose isomerase (AraA), L-ribulokinase (AraB), and L-ribulose-5-phosphate-4-epimerase (AraD) [9–13]. Additional overexpression of *S. cerevisiae* genes encoding enzymes of the non-oxidative pentose phosphate pathway (RPE1, RKL1, TAL1, and TKL1) strongly improved rates of D-xylose and L-arabinose fermentation [12, 14]. In *S. cerevisiae* strains whose metabolic pathways have been intensively optimized for pentose fermentation by metabolic and evolutionary engineering, uptake of L-arabinose and D-xylose is an important rate-controlling step [15–17].

Several *S. cerevisiae* plasma membrane hexasenote transporter proteins are able to transport D-xylose and/or L-arabinose but invariably exhibit a high *K*~m~ for these pentoses [18–25]. This low affinity causes sluggish pentose conversion (‘tailing’) towards the end of anaerobic batch cultures. Amongst the set of 18 *S. cerevisiae* hexasenote transporters (Hxt1-17 and Gal2), only the galactose transporter Gal2 and with, much lower activities, Hxt9 and Hxt10 support L-arabinose import [18, 19]. Gal2 has a high affinity for D-glucose and galactose but its affinity for L-arabinose is low (*K*~m~ = 57–371 mM) [19, 26]. Consequently, engineered strains in which L-arabinose transport depends on Gal2 fail to grow at low L-arabinose concentrations [19]. Moreover, even when D-glucose-induced transcriptional repression of *GAL2* [27–29] is prevented, kinetic competition prevents L-arabinose consumption by such strains in the presence of D-glucose.

So far, few heterologous L-arabinose transporters have been functionally expressed and characterized in *S. cerevisiae* [19, 26, 30]. In these previous studies, *S. cerevisiae* strains harbouring a functional L-arabinose fermentation pathway but no native hexose transporters proved to be excellent platforms for characterization of heterologous L-arabinose transporters. In such experiments, transporters from the yeasts Scheffersomyces stipitis (SSAraT), Pichia guilliermondii (PgAxt1) and from the plant Arabidopsis thaliana (AtStp2) were shown to support L-arabinose transport in *S. cerevisiae*. These transporters exhibited *K*~m~ values of 0.13–4.5 mM but low transport capacities, whilst also exhibiting severe D-glucose inhibition [19, 26]. Inhibition by D-xylose was only studied for PgAxt1, where it completely blocked L-arabinose uptake [26]. Conversely, L-arabinose transporters from the fungi Neurospora crassa (Lat-1) and Myceliophthora thermophila (MrLat-1) supported high-capacity, low-affinity (*K*~m~ = 58 and 29 mM, respectively) L-arabinose uptake and were also strongly affected by D-glucose inhibition [30]. The strong inhibition of these transporters by D-glucose and/or D-xylose precludes the simultaneous utilization of D-glucose and L-arabinose in *S. cerevisiae* strains depending on these transporters for L-arabinose uptake.

The filamentous fungus Penicillium chrysogenum and its genome have been intensively studied in relation to its role in the production of β-lactam antibiotics [31, 32]. *P. chrysogenum* is able to hydrolyse arabinoxylan to L-arabinose by its Axs5 extracellular arabinoferanohydrolase, followed by uptake and metabolism of L-arabinose as a carbon and energy source [33–35]. This ability implies the presence of one or more membrane transporters capable of importing L-arabinose across the plasma membrane of this fungus.

The goal of this study was to explore the *P. chrysogenum* genome for L-arabinose transporters that can be functionally expressed in *S. cerevisiae* and support D-glucose- and D-xylose-insensitive, high-affinity transport of L-arabinose. To this end, transcriptomes of L-arabinose-, ethanol- and D-glucose-limited chemostat cultures of *P. chrysogenum* were compared, and putative L-arabinose transporter genes were tested for their ability to support L-arabinose transport upon expression in an *S. cerevisiae* strain engineered for L-arabinose fermentation in which GAL2 had been deleted. A *P. chrysogenum* transporter identified in this screen, *PcAraT*, was subjected to more detailed analysis, including kinetic sugar-uptake studies with radiolabelled substrates, in vivo studies on uptake inhibition, and physiological studies with engineered *S. cerevisiae* strains in L-arabinose-limited chemostat cultures.
Methods
Microbial strains, growth media and maintenance
All S. cerevisiae strains constructed and used in this study (Table 1) are derived from the CEN.PK lineage [36]. Yeast strains were grown on synthetic medium (SM) [37] or on YP medium (10 g L⁻¹ Bacto yeast extract, 20 g L⁻¹ Bacto peptone). For shake flask cultures on synthetic medium, ammonium sulfate was replaced with urea as nitrogen source to minimize acidification. The resulting SM-urea contained 38 mmol L⁻¹ urea and 38 mmol L⁻¹ K₂SO₄ instead of (NH₄)₂SO₄. SM and YP media were autoclaved at 121 °C for 20 min, or filter-sterilized using 0.2-μm bottle-top filters (Thermo Scientific, Waltham MA). Subsequently, synthetic media were supplemented with 1 mL L⁻¹ of a sterile-filtered vitamin solution [37]. SM, SM-urea and YP media were further supplemented with 20 g L⁻¹ D-glucose or L-arabinose, by adding concentrated solutions autoclaved at 110 °C for 20 min, yielding SMD or SMA, SMD-urea or SMA-urea and YPD or YPA, respectively. Yeast cultures were grown in 100 mL medium in 500-mL shake flasks at 30 °C and at 200 rpm in an Innova Incubator (New Brunswick Scientific, Edison NJ). Solid SMD, SMA, YPD and YPA contained 1.5% Bacto agar and when indicated, 200 mg L⁻¹ G418 (Inviogen, San Diego, CA). Solid medium with ethanol and glycerol as carbon source (YPEG, SMEG, YPEG-G418) contained 2% ethanol and 3% glycerol. Selection and counter selection of the amdSYM marker cassette were performed as described previously [38]. Escherichia coli strains were grown in 5 mL Lysogeny Broth (10 g L⁻¹ Bacto tryptone, 5 g L⁻¹ Bacto yeast extract, 5 g L⁻¹ NaCl) supplemented with 100 mg L⁻¹ ampicillin in 25-mL shake flasks at 37 °C and 200 rpm in an Innova 4000 shaker (New Brunswick Scientific). Before storage at −80 °C, yeast and E. coli cultures were mixed with glycerol (30% v/v). P. chrysogenum DS17690 was kindly provided by DSM Anti-infectives (Delft, The Netherlands) and grown in mineral medium (pH 5.5), containing 3.5 g (NH₄)₂SO₄, 0.8 g KH₂PO₄, 0.5 g MgSO₄·7H₂O and 10 mL of trace element solution (15 g L⁻¹ Na₂EDTA-2H₂O,

| Strain | Relevant genotype | References |
|--------|-------------------|------------|
| CEN.PK 113-7D | MATαura3his3leu2trp1mal2-8cSUC2 | [36] |
| CEN.PK 113-5D | MATαura3-s2his3leu2trp1mal2-8cSUC2 | [36] |
| CEN.PK102-12A | MATαura3-s2his3-d1leu2-3,112trp1mal2-8cSUC2 | [36] |
| IMX080 | CEN.PK102-12Agal1::sphis5, hxs1::KLEU2 | [75] |
| IMXS31 | CEN.PK113-5dcan1::cas9-natNT2 | This study |
| IMX486 | IMX080gal1::cas9-amsSYM | This study |
| IMX604 | IMX486ura3-s2 gre3::PTDH3-RPE1, pPGK1-TKL1, pTEF1-TAL1, pPG11-NQM1, pTP1I-RK1, pPYK1-TKL2 | This study |
| IMX658 | IMX604ura3-s2gal80::(pTPI-AraA-tCyc1)*9, pPYK1-AraB-tPG1, pPGK1-AraD-tDH3 | This study |
| IMX660 | IMX658hxs2::KURA3 | This study |
| IMX728 | IMX658hxs2::PCaraT | This study |
| IMX844 | IMX660gal2::KanMX | This study |
| IMX869 | IMX728gal2::KanMX | This study |
| IMX918 | IMXS31gre3::PTDH3-RPE1, pPGK1-TKL1, pTEF1-TAL1, pPG11-NQM1, pTP1I-RK1, pPYK1-TKL2 | This study |
| IMX928 | IMX918gal80::(pTPI-AraA-tCyc1)*9, pPYK1-AraB-tPG1, pPGK1-AraD-tDH3 | This study |
| IMX929 | IMX918gal80::(pTPI-AraA-tCyc1)*9, pPYK1-AraB-tPG1, pPGK1-AraD-tDH3, pUEDE348 | This study |
| IMX1504 | IMX928, gal2Δ, pUDR245 | This study |
| IMX1505 | IMX928gal2::pADH1-Pc1.3g04640-tpMA1 (from pPW111), pUDR245 | This study |
| IMX1506 | IMX928gal2::pADH1-Pc1.3g08230-tpMA1 (from pPW113), pUDR245 | This study |
| IMX1507 | IMX928gal2::pADH1-Pc1.6g05670-tpMA1 (from pPW116), pUDR245 | This study |
| IMX1508 | IMX928gal2::pADH1-Pc20g01790-tpMA1 (PcaraT) (from pPW118), pUDR246 | This study |
| IMX1509 | IMX928gal2::pADH1-Pc22g14520-tpMA1 (from pPW123), pUDR245 | This study |
| DS68616 | MATα, ura3-s2, leu2-112, gre3::loxP, loxPrtp-tPPI-TAL1, loxP-tPPI-RK1, loxP-tPPI-RPE1, leu2::pADH1-XXX1-1Cyc1-EU2, ura3::URA3-tPPI-yyy4-tCyc1 | DSM, The Netherlands |
| DS68625 | DS68616his3::loxP, htx2::loxP-kmx-loxP, htx367::loxP-hphMX-loxP, htx145::loxP-natMX-loxP, gal2::loxP-zeoMX-loxP | [45] |
| DS68625-PcaraT | DS68625, pRS313-PcaraT | This study |
| DS68625-GAL2 | DS68625, pRS313-GAL2 | This study |
| DS68625-mcs | DS68625, pRS313-mcs (empty) | This study |
0.5 g L\(^{-1}\) Cu\(_2\)SO\(_4\)·5H\(_2\)O, 2 g L\(^{-1}\) ZnSO\(_4\)·7H\(_2\)O, 2 g L\(^{-1}\) MnSO\(_4\)·H\(_2\)O, 4 g L\(^{-1}\) FeSO\(_4\)·7H\(_2\)O, and 0.5 g L\(^{-1}\) CaCl\(_2\)·2H\(_2\)O) per litre of demineralized water. The mineral medium was supplemented with 7.5 g L\(^{-1}\) d-glucose. Precultivation for chemostat cultures was carried out on mineral medium with 7.5 g L\(^{-1}\) d-glucose, 7.5 g L\(^{-1}\) L-arabinose, or 5.8 g L\(^{-1}\) ethanol as carbon source.

**Molecular biology techniques**

DNA fragments were amplified by PCR amplification with Phusion Hot Start II High Fidelity Polymerase (Thermo Scientific) and desalted or PAGE-purified oligonucleotide primers (Sigma-Aldrich, St. Louis, MO) performed according to the manufacturers’ instructions. Diagnostic PCRs were run with DreamTaq polymerase (Thermo Scientific). Oligonucleotide primers used in this study are listed in Additional file 1. PCR products were separated by electrophoresis on 1% (w/v) agarose gels (Thermo Scientific) in TAE buffer (Thermo Scientific) and, if required, purified with a Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA) or a GenElute PCR Clean-Up kit (Sigma-Aldrich). Yeast or *E. coli* plasmids were isolated with a Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research), or a GenElute Plasmid Miniprep kit (Sigma-Aldrich), respectively. A YeaStar Genomic DNA kit (Zymo Research) or an SDS/lithium acetate protocol [39] was used to isolate yeast genomic DNA. Yeast strains were transformed using the lithium acetate protocol [39], with oligonucleotide primers listed in Additional file 1. pUDE348 was derived from pMEL10 by first PCR amplifying the plasmid backbone using primers 5792 and 5980. The gRNA sequence was introduced in the gRNA expression cassette with primers 6631 and 5979 using pMEL10 [44] as a template. Subsequently, both fragments were combined using the Gibson Assembly Cloning kit. pUD405 was obtained by integration of a Gal2-flanked KanMX cassette obtained from pUG6 with primers 944 and 945 into a pET1.2 blunt vector according to the manufacturers’ instructions. Construction of the low-copy-number centromeric plasmid pRS313-mcs was described previously [45]. GAL2 was amplified from genomic DNA of *S. cerevisiae* DS68616 [45] and *PcarAT* was amplified from plasmid pPWT118 using primers F *GAL2* and R *GAL2* Cfr9i and primers F *PcarAT* Xbai and R *PcarAT* Cfr9i, respectively, and cloned into pRS313-mcs, resulting in plasmids pRS313-*PcarAT* and pRS313-*GAL2*.

**Strain construction**

Gene expression cassettes were PCR amplified with oligonucleotide primers shown in Additional file 1 and genomic DNA of CEN.PK113-7D or plasmids described in Table 2. Gene knock-outs and construct integrations were introduced with a chimeric CRISPR/Cas9 editing system [44]. To enable CRISPR/Cas9 mediated editing in strain IMX080, the SpCas9 expression cassette was amplified from p414-4TEF1-1as9-tCYC1 (Addgene plasmid # 43802) and integrated into the GAL1 locus via in vivo assembly, together with the amdSYM marker, yielding strain IMX486. For overexpression of the non-oxidative pentose phosphate pathway (PPP), IMX486 and IMX581 were co-transformed with gRNA plasmid pUDE335 and repair fragments flanked with either 60 bp homologous to GRE3 or with synthetic tags [46] assisting homologous recombination of the PPP expression cassettes (gre\(_3\)flank-pTDH3-RPE1-TagH, TagH-pPGK1-TKL1-Tagl, Tagl-pTEF1-TAL1-TagA, TagA-pPGI1-NQM1-TagB, TagB-pTP1I-RKI1-TagC, TagC-pPYK1-TKL2-gre\(_3\)flank). After counter selection of the URA3-based plasmid pUDE335, the resulting strains, IMX604 and IMX918, respectively, were co-transformed
with pUDE348 and repair fragments flanked with either 60 bp homologous to GAL80 or with synthetic tags [46] (GAL80 blind-pPTPI-araA-TagG, TagG-pPTPI-araA-TagA, TagA-pPTPI-araA-TagB, TagB-pPTPI-araA-TagC, TagC-pPTPI-araA-TagD, TagD-pPTPI-araA-TagM, TagM-pPTPI-araA-TagN, TagN-pPTPI-araA-TagO, TagO-pPTPI-araA-TagI, TagI-pPYK1-araB-TagK, TagK-pPGK1-araD-GAL80 blind) resulting in nine copies of araA and a single copy of araB and araD integrated in the GAL80 locus. After verification of the resulting strains IMX929 and IMX658, respectively, plasmid pUDE348 was counter selected in IMX929 to yield strain IMX928. Disruption of HXK2 in IMX658 was done by PCR amplification and transformation of the KIURA3-based deletion cassette from pUG-72 [76] to obtain strain IMX660 upon transformation and plating in solid SMA. GAL2 was disrupted in IMX660 by transformation with a KanMX cassette amplified from pUD405 with primers 944 and 945 flanked with 60 bp homologous to GAL2. Transformants were incubated for 2 h in YPE before plating on YPEG-G418, yielding strain IMX844. Expression of PcaraT in IMX658 was achieved by transforming IMX658 with the gRNA plasmid pUDE327 together with an expression cassette of PcaraT (pADH1-PcaraT-tPMA1) with flanking regions homologous to the HXK2 locus amplified with the primer pair 7660 and 7676. Counter selection of the pUDE327 and subsequent transformation of a DNA fragment derived from CEN. PK113-7D using primers 2641 and 1522 repaired uracil auxotrophy and resulted in strain IMX728. GAL2 was disrupted in IMX728 by transformation with a KanMX cassette amplified from pUD405 with primers 944 and 945 flanked with 60 bp homologous to GAL2. Transformants were incubated for 2 h in YPE before plating on YPEG-G418, yielding strain IMX869. Strains IMX1505-1509 were constructed by co-transforming pUDR245 or pUDR246 and a GAL2-flanked expression cassette (pADH1-ORF-tPMA1) amplified from pPWT111, 113,
116, 118 or 123, respectively, amplified with the primer pair 10585 and 10584. IMX1504, harbouring a knock-out of GAL2, was constructed by co-transforming pUJR245 and a repair fragment based on the annealed primers 9563 and 9564. Transformation of GAL2 and *PceaT* plasmids, and the pRS313-mcs plasmid (as an empty plasmid/control) into the hexose-transporter deletion strain DS68625 yielded strains DS68625-GAL2, DS68625-*PceaT*, and DS68625-mcs.

**Growth experiments in shake flasks**

Thawed 1-mL aliquots from frozen stock cultures were used to inoculate shake flask precultures on SM-urea supplemented with either **d-glucose** (20 g L\(^{-1}\)), **l-arabinose** (20 g L\(^{-1}\)), or both sugars (both 20 g L\(^{-1}\)). These precultures were used to inoculate a second culture which was subsequently used to inoculate a third culture which was inoculated at an initial OD\(_{660}\) of 0.1 and used to monitor growth. Optical densities at 660 nm were measured with a Libra S11 spectrophotometer (Biochrom, Cambridge, United Kingdom). Maximum specific growth rates (\(\mu_{max}\)) were derived from at least four consecutive data points derived from samples taken during the exponential growth phase of each culture.

**Spot plates**

**l-arabinose**-metabolizing *S. cerevisiae* strains expressing putative *P. chrysogenum* l-arabinose transporter genes (IMX1504-1509) were grown on SMD medium and a total number of approximately \(10^4\), \(10^3\), \(10^2\), and \(10^1\) cells were spotted on duplicate agar plates as described previously \([47, 48]\) containing either 20 g L\(^{-1}\) **l-arabinose** or **d-glucose** as carbon source (pH 6). Cell numbers were estimated from calibration curves of OD\(_{660}\) versus cell counts determined with an Accuri flow cytometer (Becton–Dickinson B.V., Breda, The Netherlands), derived from exponentially growing shake flask cultures of *S. cerevisiae* CEN.PK113-7D on SMD medium. SMA and SMD plates were incubated at 30 °C for 97 and 41 h, respectively.

**Chemostat cultivation**

Aerobic carbon-limited chemostat cultures of *P. chrysogenum* were grown at 25 °C in 3-L turbine-stirred bioreactors (Applikon, Schiedam, The Netherlands) with a working volume of 1.8 L and a dilution rate of 0.03 h\(^{-1}\) as described previously \([49]\), with the exception that, in addition to cultures grown on 7.5 g L\(^{-1}\) **d-glucose**, chemostat cultures were also grown on either 7.5 g L\(^{-1}\) **l-arabinose** or 5.8 g L\(^{-1}\) ethanol. Aerobic, **l-arabinose**-limited chemostat cultures of *S. cerevisiae* were grown at 30 °C in 2-L Applikon bioreactors with a working volume of 1 L and at a dilution rate of 0.05 h\(^{-1}\). SMA (7.5 g L\(^{-1}\) **l-arabinose**) supplemented with 0.15 g L\(^{-1}\) Pluronic antifoam PE 6100 was used as culture medium for the initial batch phase and for chemostat cultivation, with the exception of the initial batch phase of strain IMX929 which was grown on 20 g L\(^{-1}\) **l-arabinose**. Cultures were stirred at 800 rpm, kept at pH 5.0 by automatic addition of 2 M KOH, and sparged with 0.5 L min\(^{-1}\) air. Upon completion of the batch phase, chemostat cultivation was initiated, ensuring a constant culture volume with an electric level sensor. When after at least five volume changes, biomass dry weight and CO\(_2\) production varied by less than 2% over two consecutive volume changes, the culture was considered to be in steady state.

**Analytical methods**

*Penicillium chrysogenum* biomass dry weight was determined in duplicate by filtration of 10 mL culture sample over pre-weighed glass fibre filters (Type A/E, Pall Life Sciences, Hoegaarden, Belgium). After filtration, filters were washed with demineralized water and dried for 10 min at 600 W in a microwave oven (Bosch, Stuttgart, Germany) prior to reweighing. Biomass dry weight in *S. cerevisiae* culture samples was determined with a similar procedure using nitrocellulose filters (0.45-µm pore size; Gelman Laboratory, Ann Arbor, MI) and drying for 20 min in a microwave oven at 360 W output. Optical density (OD) of the cultures was determined at 660 nm with a Libra S11 spectrophotometer (Biochrom, Cambridge, United Kingdom). Determination of CO\(_2\) and O\(_2\) concentrations in the bioreactor exhaust gas and HPLC analysis of metabolite concentrations in culture supernatant samples were performed as described previously \([50]\).

**Sampling, RNA extraction, microarrays analysis, and data analysis**

Samples (60 mL) from *P. chrysogenum* chemostat cultures were rapidly filtered over a glass fibre filter (Type A/E, Pall Life Sciences) and further processed for total RNA extraction by phenol–chloroform extraction \([49]\). The cRNA sample preparation (cDNA synthesis, purification, in vitro transcription, labelling, purification, fragmentation and biotinylation) was performed according to Affymetrix recommendations \([31]\). Eventually cRNA samples were hybridized onto custom-made *P. chrysogenum* GeneChip microarrays (array code DSM_PENA52025F). Data acquisition, hybridization, quantification of processed array images, and data filtering were performed using the Affymetrix GeneChip Operating Software (GCOS version 1.2). Global array normalization was performed by scaling the global fluorescence intensity of each microarray to 100. The scaling factors of the individual arrays were highly similar and ranged from 0.21
to 0.35. Subsequently, significant variations in expression were statistically estimated by comparing replicate array experiments using the Significance Analysis of Microarray software (SAM version 2.0) [51] with the multiclass setting. A false discovery rate of 1% was applied to minimize the chance of false-positive hits. Genes with an over threefold higher transcript level in arabinose-grown cultures than in d-glucose-grown cultures and a less than threefold difference in ethanol- and d-glucose-grown cultures were deemed to show arabinose-specific expression. Transcriptome data of strain DS17690 grown on d-glucose, ethanol or arabinose are accessible at NCBI Genome Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under Accession Numbers GSE12632, GSE24212 and GSE10449, respectively [49].

Analysis of sugar uptake kinetics

Uptake experiments with [14C] l-arabinose, [14C] d-xylose, or [14C] d-glucose, labelled at the first carbon atom (50–60 mCi/mmol) (ARC St. Louis, MO), were performed with S. cerevisiae hexose-transporter deletion strains (DS68625) harbouring a low copy plasmid with constitutively expressed PearaT (pRS313-PearaT) or GAL2 (pRS313-GAL2). The experimental workflow was carried out as described previously [45] with [14C] l-arabinose concentrations of 0.5–2000 mmol L⁻¹, [14C] d-xylose concentrations of 0.5–500 mM, or [14C] d-glucose concentrations of 0.1–500 mmol L⁻¹. Transport competition experiments were carried out in the presence of 50 mmol L⁻¹ [14C] l-arabinose and 0–500 mmol L⁻¹ d-glucose or d-xylose, and at [14C] l-arabinose concentration of 2 mmol L⁻¹ together with increasing d-glucose and xylose concentrations of 0–20 mM. Maximum biomass-specific transport rates (Vₘₐₓ) calculated from transport assays were expressed as nmol sugar transported per milligram biomass dry weight per minute [nmol (mg biomass)⁻¹ min⁻¹]. As this Vₘₐₓ is influenced by the expression level of the relevant transporter, it is not solely dependent on intrinsic transporter kinetics. The impact of proton-gradient uncoupling on transport activity was determined in 200 μL synthetic medium at a [¹³C] l-arabinose concentration of 2 mmol L⁻¹, by comparing transport rates upon addition of either 10 μmol L⁻¹ CCCP (0.5 μL of a stock solution dissolved in 100% DMSO), 0.5 μL DMSO (control), or 0.5 μL water.

Phylogenetic methods

Protein sequences used for generation of a phylogenetic tree were derived from NCBI (https://www.ncbi.nlm.nih.gov/) and the Saccharomyces Genome Database (https://www.yeastgenome.org/). Mafft was used to generate a CLUSTAL format alignment of all sequences, using the L-INS-i method default settings (https://mafft.cbrc.jp/alignment/server/) [52, 53]. Alignments were further processed using neighbour-joining and a 500 times bootstrap. The resulting Newick tree file was visualized and midpoint rooted in iTOL (https://itol.embl.de/) [54]. Gene accession numbers were ScGAL2: P13181, Pecara1: CAP85508, Ssara1: XP_001382755, Atspa2: OAP13698, Knax1: GZ791039, Pgax1: GZ791040, Amlat1: AY923868, Amlat2: AY923869, Nclat-1: EAA30346, Mtlat-1: XP_003663698.

Results

Chemostat-based transcriptome analysis of P. chrysogenum for identification of possible l-arabinose transporter genes

Filamentous fungi exhibit a much broader range of carbon source utilization than S. cerevisiae and, similar to many other ascomycetous fungi, P. chrysogenum can grow on l-arabinose as the sole carbon source [33, 55]. To identify candidate structural genes for l-arabinose transporters in P. chrysogenum, carbon-limited chemostat cultures of strain DS17690 were grown at a dilution rate of 0.03 h⁻¹ on different carbon sources. To discriminate between alleviation of carbon repression and l-arabinose induction, duplicate d-glucose-, l-arabinose-, and ethanol-limited chemostat cultures were performed. RNA was extracted from steady-state cultures and gene expression levels were obtained using Affymetrix DNA-arrays [49]. A total of 540 genes were differentially expressed over the three conditions. Of these differentially expressed genes, 137 exhibited an over threefold higher transcript level in l-arabinose-limited cultures than in d-glucose-limited cultures, as well as a less than threefold difference in transcript level between ethanol- and d-glucose-limited cultures (Additional file 2). Genes whose transcript levels in l-arabinose- and ethanol-limited cultures were both at least twofold higher than in d-glucose-grown cultures were not considered for further analysis as their regulation could have reflected unspecific d-glucose (de)repression. An annotation screen indicated that 16 of the identified ‘arabinose-induced’ genes encoded putative l-arabinose transporters, whose transcript levels were at least 30-fold higher in l-arabinose-limited cultures than in the d-glucose-limited cultures (Table 3). Five of these genes, whose transcript levels were at least 30-fold higher in l-arabinose-limited cultures than in d-glucose-limited cultures, shared similarity with the S. cerevisiae maltose transporter Mal31, the N. crassa d-glucose transporter Rco-3, the Kluyveromyces lactis high-affinity d-glucose transporter Hgt1 and the S. cerevisiae allantoinate transporter Dal5. These five transporter genes (Pc13g08230, Pc16g05670, Pc20g01790, Pc22g14520, and Pc13g04640, respectively) were selected for further functional analysis.
Table 3 Putative transporter genes that showed higher relative transcript levels in aerobic, L-arabinose-limited chemostat cultures of *Penicillium chrysogenum* than in corresponding d-glucose- and ethanol-limited cultures

| Gene                | Strong similarity to | Relative transcript levels under different nutrient limitations |
|---------------------|----------------------|---------------------------------------------------------------|
|                     |          | Glucose | L-Arabinose | Ethanol | Ethanol versus glucose (ratio) | L-Arabinose versus glucose (ratio) |
| Pc13g08230          | S. cerevisiae maltose transport protein Mal31 | 13 ± 1 | 664 ± 3 | 17 ± 1 | 1.4 | 53 |
| Pc16g05670          | Neurospora crassa glucose transporter rco-3 | 63 ± 28 | 3176 ± 40 | 69 ± 1 | 1.1 | 51 |
| Pc20g01790 (Pcan07) | Klyveromyces lactis high-affinity glucose transporter HGT1 | 32 ± 6 | 1415 ± 42 | 46 ± 3 | 1.4 | 44 |
| Pc22a14520          | S. cerevisiae allantoate permease Dal5 | 19 ± 2 | 770 ± 104 | 28 ± 1 | 1.5 | 41 |
| Pc13g04640          | K. lactis high-affinity glucose transporter HGT1 | 29 ± 5 | 971 ± 32 | 53 ± 7 | 1.8 | 34 |
| Pc21g01910          | K. lactis high-affinity glucose transporter HGT1 | 12 ± 1 | 167 ± 26 | 12 ± 1 | 1.0 | 14 |
| Pc12g00190          | Candida albicans ABC transporter CDR4 | 13 ± 2 | 164 ± 24 | 29 ± 2 | 2.2 | 12 |
| Pc14g01680          | Escherichia coli L-fucose permease fucP | 106 ± 14 | 1269 ± 172 | 68 ± 1 | 0.64 | 12.0 |
| Pc21g12210          | Aspergillus nidulans quinate transporter protein qutD | 12 ± 0 | 118 ± 1 | 12 ± 1 | 1 | 9.8 |
| Pc06g01480          | S. cerevisiae maltose transport protein Mal31 | 459 ± 85 | 3551 ± 102 | 226 ± 3 | 0.5 | 7.7 |
| Pc13g10030          | S. cerevisiae high-affinity nicotinic acid permease Tna1 | 125 ± 25 | 827 ± 33 | 216 ± 3 | 1.7 | 6.6 |
| Pc21g09830          | K. lactis high-affinity glucose transporter HGT1 | 185 ± 9 | 842 ± 1 | 126 ± 3 | 0.68 | 4.6 |
| Pc16g02680          | S. cerevisiae allantoate permease Dal5 | 80 ± 29 | 360 ± 14 | 113 ± 6 | 1.4 | 4.5 |
| Pc12g05440          | S. cerevisiae maltose transport protein Mal31 | 596 ± 201 | 2633 ± 64 | 104 ± 8 | 0.17 | 4.4 |
| Pc13g15590          | S. cerevisiae glucose permease Rgt2 | 12 ± 1 | 480 ± 10 | 12 ± 1 | 1 | 4.0 |
| Pc13g06440          | S. cerevisiae high-affinity nicotinic acid permease Tna1 | 66 ± 13 | 225 ± 11 | 48 ± 5 | 0.73 | 3.4 |

*P. chrysogenum* DSM1769 was grown in L-arabinose-, d-glucose-, or ethanol-limited chemostat cultures (dilution rate = 0.03 h⁻¹, pH 6.5, T = 25 °C). Underlined genes were selected for further analysis based on a ≥30-fold higher transcript level in L-arabinose-limited cultures than in d-glucose-limited cultures. Data represent average ± mean deviation of globally scaled (target 100) Affymetrix microarrays for independent duplicate chemostat cultures.

**PcAraT:** a *P. chrysogenum* L-arabinose transporter that can be functionally expressed in *S. cerevisiae*

*Saccharomyces cerevisiae* strains in which HXT transporter genes have been deleted and which express heterologous pathways for pentose metabolism have proven to be powerful platforms for screening and characterization of heterologous pentose transporter genes [19, 26, 56]. To enable screening for *P. chrysogenum* L-arabinose transporters, *S. cerevisiae* strains were first engineered for L-arabinose consumption. Using CRISPR/Cas9-mediated in vivo assembly [44], the overexpression cassettes for all structural genes involved in the non-oxidative pentose phosphate pathway (*TAL1, NQM1, TKL1, TKL2, RKI1, RPE1*) were stably integrated into the GRE3 locus, thereby inactivating synthesis of the Gre3 aldose reductase. Subsequently, nine copies of an expression cassette for overexpression of codon-optimized *L. plantarum* L-arabinose isomerase AraA and single copies of *L. plantarum* AraB (L-ribulokinase) and AraD (L-ribulose-5-phosphate-4-epimerase) expression cassettes were integrated into the *GAL80* locus, using a strain construction strategy previously described for expression of a d-xylose pathway into *S. cerevisiae* [50]. This integration inactivated *GAL80* and thereby alleviated transcriptional repression by d-glucose of *GAL2*, which encodes the major L-arabinose transporter in *S. cerevisiae* [57, 58]. The resulting strain IMX929 was able to grow in liquid media supplemented with L-arabinose as the sole carbon source and was used as a platform strain to test if any of the five selected putative *P. chrysogenum* transporter genes, placed under the control of the constitutive *ADH1* promoter, could support L-arabinose transport in *S. cerevisiae*. To this end, single copies of codon-optimized expression cassettes were integrated into the *GAL2* locus of the L-arabinose-metabolizing *S. cerevisiae* strain IMX928, a uracil auxotrophic daughter strain of IMX929, thereby inactivating the *GAL2* gene. Consistent with previous studies [19, 26], inactivation of *GAL2* in the L-arabinose metabolizing strain IMX928 yielded a strain (IMX1504) that was unable to grow on SMA plates (Fig. 1). All five strains in which *GAL2* had been replaced by putative *P. chrysogenum* transporter genes (IMX1505-1509) showed vigorous growth on SMD plates. However, only strain IMX1508, which expressed the *P. chrysogenum* gene Pc20g01790, showed growth on L-arabinose (Fig. 1). Based on this observation, Pc20g01790 was designated *PcaraT* (*P. 
chrysogenum Arabinose Transporter). A Blast-p search revealed strong homology of Pc20g01790 with the K. lactis gene HGT1, which encodes a high-affinity D-glucose and galactose transporter [59, 60].

PcaraT encodes a high-affinity, high-specificity L-arabinose transporter
Sugar transport kinetics of PcaraT were analysed using 14C-labelled L-arabinose, D-xylose and D-glucose. To dissect transporter kinetics of PcaraT and Gal2, their structural genes were separately expressed in S. cerevisiae DS68625 [45]. Each gene was introduced on a centromeric plasmid and expressed from the HXT7 promoter. In strain DS68625, the major hexose-transporter genes (HXT1-7 and GAL2) are deleted, whilst its inability to metabolize L-arabinose enables the specific analysis of sugar uptake rather than the combination of radioactive sugar uptake and metabolism. The negative control strain DS68625-mcs (DS68625 transformed with the ‘empty’ centromeric plasmid pRS313-mcs) did not show significant [14C] L-arabinose uptake, whilst expression of either Gal2 or PcaraT (strains DS68625-Gal2 and DS68625-PcaraT, respectively) restored L-arabinose transport (Table 4). In kinetic analyses, the $K_m$ of PcaraT for L-arabinose (0.13 mmol L$^{-1}$) was found to be three orders of magnitude lower than that of Gal2 (335 mmol L$^{-1}$), whilst its transport capacity ($V_{max}$) was 14-fold lower than that of Gal2 [5.3 and 75 nmol (mg biomass)$^{-1}$ min$^{-1}$, respectively] (Table 4). PcaraT was found to be highly L-arabinose specific, as its expression in strain DS68625 did not support transport of either [14C] D-glucose or [14C] D-xylose. Consistent with earlier reports [19, 26], expression of Gal2 in strain DS68625 enabled transport of D-glucose ($K_m = 1.9$ mmol L$^{-1}$, $V_{max} = 26$ nmol (mg biomass)$^{-1}$ min$^{-1}$), whilst Gal2 has

| Sugar | Gal2 | PcaraT |
|-------|------|--------|
| $K_m$, ARA (mmol L$^{-1}$) | 335 ± 21 | 0.13 ± 0.03 |
| $V_{max}$, ARA [nmol (mg biomass)$^{-1}$ min$^{-1}$] | 75 ± 5.2 | 5.3 ± 0.2 |
| $K_m$, GLC (mmol L$^{-1}$) | 1.9 | – |
| $V_{max}$, GLC [nmol (mg biomass)$^{-1}$ min$^{-1}$] | 26 | – |
| L-Arabinose transport inhibition by glucose | 85% | 63% |
| $K_m$, XYL (mmol L$^{-1}$) | 226 [20] | – |
| $V_{max}$, XYL [nmol (mg biomass)$^{-1}$ min$^{-1}$] | 91 [20] | – |
| L-Arabinose transport inhibition by D-xylose | 29% | 22% |

Transport inhibition was determined at 50 mmol L$^{-1}$ [14C]-l-arabinose and 100 mmol L$^{-1}$ of either D-glucose or D-xylose and expressed relative to the transport rate observed in the absence of D-xylose or D-glucose. Values are represented as average ± mean deviation of duplicate experiments. Graphs used to calculate kinetic parameters are shown in Additional files 3–6. ARA, L-arabinose; GLC, D-glucose; XYL, D-xylose; –, no transport.
previously been shown to enable low-affinity d-xylose transport ($K_m = 226 \text{ mmol L}^{-1}$; [20]).

The impact of the presence of d-glucose and d-xylose on l-arabinose transport by Gal2 and PcAraT was investigated in transport assays with 50 mmol L$^{-1}$ [14C] l-arabinose and increasing concentrations of non-radioactive d-glucose or d-xylose. In these assays, both transporters exhibited a reduced l-arabinose transport capacity in the presence of d-glucose or d-xylose (Table 4, Additional file 3). At a concentration of 100 mmol L$^{-1}$ (i.e. twice the concentration of l-arabinose), d-xylose and d-glucose inhibited l-arabinose uptake rate via Gal2 by 29 and 85%, respectively. In contrast, l-arabinose trans-d-glucose inhibited l-arabinose uptake rate via Gal2 by twice the concentration of l-arabinose, d-xylose and d-glucose. In these assays, both transporters exhibited a reduced l-arabinose transport capacity in the presence of d-glucose or d-xylose. In these assays, both transporters exhibited a reduced l-arabinose transport capacity in the presence of d-glucose or d-xylose (Table 4, Additional file 3). At a concentration of 100 mmol L$^{-1}$ (i.e. twice the concentration of l-arabinose), d-xylose and d-glucose inhibited l-arabinose uptake rate via Gal2 by 29 and 85%, respectively. In contrast, l-arabinose transport via PcAraT was less impaired at this concentration of d-xylose, and especially, d-glucose (22 and 63% inhibition, respectively). To study the transport mechanism of PcAraT, the impact of the protonophore uncoupler CCCP on transport kinetics was tested. Transport of l-arabinose via Gal2, which mediates facilitated diffusion of sugars [61], was not affected by CCCP, whilst this uncoupler completely abolished transport via PcAraT (Additional file 7). These results indicate that PcAraT mediates proton-coupled import of l-arabinose.

**Functional expression of PcaraT in an l-arabinose-fermenting S. cerevisiae strain enables l-arabinose consumption in the presence of d-glucose**

The ability to transport l-arabinose in the presence of d-glucose is a highly relevant characteristic in the construction of platform S. cerevisiae strains for conversion of lignocellulosic hydrolysates [8]. To investigate whether expression of PcaraT can confer this ability, a set of three strains was constructed that (i) could not metabolize d-glucose due to the deletion of HXK1, HXK2, GLK1 and GAL1 [20, 62]; (ii) (over)expressed non-oxidative PPP enzymes and the L. plantarum AraA, AraB and AraD genes to enable l-arabinose metabolism; and (iii) had different genotypes with respect to l-arabinose transport (GAL2, PcaraT/gal2Δ and gal2Δ in strains IMX660, IMX869 and IMX844, respectively). Since these ‘arabinose specialist strains’ cannot grow on d-glucose, the impact of the presence of d-glucose on l-arabinose metabolism can be directly measured via its effect on growth. As anticipated, strain IMX844 (gal2Δ) was unable to grow on synthetic medium supplemented with either 20 g L$^{-1}$ l-arabinose or a mix of 20 g L$^{-1}$ of each, l-arabinose and d-glucose. In contrast, the l-arabinose specialist strains IMX660 (GAL2) and IMX869 (PcaraT/gal2Δ) grew on synthetic medium with l-arabinose as the sole carbon source at specific growth rates of 0.240 ± 0.001 and 0.099 ± 0.001 h$^{-1}$, respectively (Fig. 2a). However, when 20 g L$^{-1}$ d-glucose was added to the l-arabinose medium, strain IMX660 (GAL2) did not show growth during a 120-h batch cultivation experiment (Fig. 2b), whilst strain IMX869 (PcaraT/gal2Δ) grew at 60% of the specific growth rate observed in the absence of d-glucose ($\mu = 0.057 \pm 0.003$ h$^{-1}$ versus 0.099 ± 0.001 h$^{-1}$, Fig. 2b). This result indicated that expression of PcaraT in strain IMX869 enabled uptake of l-arabinose in the presence of d-glucose.

**Low residual substrate concentrations in chemostat cultures confirm high-affinity l-arabinose transport kinetics of PcaraT**

To further evaluate the in vivo impact of l-arabinose transport via PcaraT, biomass-specific l-arabinose consumption in the presence of d-glucose was investigated by deletion of the hexose kinase genes HXK1, HXK2, GLK1 and GAL1, and expressing either GAL2 (IMX660, filled circles) or the P. chrysogenum transporter PcaraT (IMX869, open circles) as the sole l-arabinose transporter. To assess the ability of Gal2 and PcaraT to support import of l-arabinose by growing cultures in the absence (a) and presence (b) of d-glucose, specific growth rates were estimated from shake flask cultures on synthetic media supplied with 20 g L$^{-1}$ l-arabinose (a) and on synthetic media supplied with l-arabinose and d-glucose (20 g L$^{-1}$ each, b).

![Fig. 2](image-url)
consumption rates and residual substrate concentrations were analysed in l-arabinose-limited, aerobic chemostat cultures, grown at a dilution rate of 0.05 h\(^{-1}\). Under these conditions, the l-arabinose-metabolizing strain IMX1508 (PcaraT/gal2Δ) exhibited a residual l-arabinose concentration of only 4.2 × 10\(^{-3}\) g L\(^{-1}\), compared to 1.8 g L\(^{-1}\) in cultures of strain IMX929 (GAL2) (Table 5). In these growth experiments, different promoters were used for expression of PcaraT and GAL2 (pADH1 and derepressed pGAL2, respectively). However, whilst this may moderately affect expression levels of the two transporters, this cannot explain the over 1000-fold difference in residual l-arabinose concentration. This difference was entirely consistent with the conclusion from the kinetic analyses of \(^{14}\)C-l-arabinose uptake, in which both transporter genes were expressed from the same promoter (pHX7) and which also indicated that PcaraT encodes an l-arabinose transporter with a much higher affinity for l-arabinose than Gal2. In shake flask batch cultures grown on an initial l-arabinose concentration of 7.5 g L\(^{-1}\), these strains exhibited initial specific growth rates of 0.085 and 0.13 h\(^{-1}\), respectively. Based on this observation and on the high \(K_m\) of Gal2 for l-arabinose ([19, 25], this study), the in vivo activity of PcaraT can be expected to exceed that of Gal2 when l-arabinose concentrations are below ca. 4 g L\(^{-1}\).

In duplicate steady-state chemostat cultures, the biomass-specific l-arabinose consumption rate of strain IMX1508 (PcaraT) was approximately 14% higher than the one of strain IMX929 (GAL2; 0.8 ± 0.1 and 0.7 ± 0.1 mmol g\(^{-1}\) h\(^{-1}\)), reflecting the slightly lower biomass yield of the former strain. This difference in biomass yield is close to the difference of 8.1% that, based on published estimates of the P/O ratio and proton stoichiometry of the plasma membrane ATPase in aerobic \textit{S. cerevisiae} cultures (both close to 1.0, [63, 64]), would be expected if l-arabinose uptake via PcaraT occurred via symport with a single proton.

### Table 5 Physiological data derived from steady-state chemostat cultures of engineered l-arabinose-metabolizing \textit{S. cerevisiae} strains

|                     | IMX929 (GAL2) | IMX1508 (PcaraT) |
|---------------------|---------------|------------------|
| Residual l-arabinose (g L\(^{-1}\)) | 1.77 ± 0.19   | 0.004 ± 0.002   |
| \(Y_{X/S}\) (g biomass (g l-arabinose)\(^{-1}\)) | 0.48 ± 0.06   | 0.40 ± 0.01     |
| \(q_{\text{l-arabinose}}\) (mmol g\(^{-1}\) h\(^{-1}\)) | 0.70 ± 0.10   | 0.80 ± 0.08     |

Strains expressing either GAL2 (IMX929) or PcaraT (IMX1508) as the sole functional l-arabinose transporter were grown in aerobic, l-arabinose-limited chemostat cultures (7.5 g L\(^{-1}\) l-arabinose, dilution rate = 0.05 h\(^{-1}\), pH 5, \(T = 30^\circ\)C). Data are derived from independent triplicate experiments and presented as average ± mean deviation.

### Discussion

Chemostat-based transcriptome analysis of \textit{P. chrysogenum} proved to be an efficient method to identify candidate genes for l-arabinose transporters in this fungus. In comparison with similar studies in batch cultures, use of chemostat cultures offered several advantages. First, chemostat cultivation at a fixed dilution rate eliminated the impact of specific growth rate on transcriptional regulation [65]. Furthermore, use of l-arabinose-limited chemostat cultures of \textit{P. chrysogenum}, in which residual concentrations of this pentose were very low, enabled a focus on the identification of high-affinity transporters. Finally, the use of both \(\alpha\)-glucose- and ethanol-limited cultures as references helped to eliminate transcriptional responses of \textit{P. chrysogenum} that were specific to either of these two carbon sources, e.g. as a result of CreA-mediated \(\alpha\)-glucose repression of relevant transporter genes [66, 67]. Although this study was focused on l-arabinose transport, the \textit{P. chrysogenum} transcriptome dataset from \(\alpha\)-glucose, ethanol and arabinose grown cultures generated in this study [available via GEO, (https://www.ncbi.nlm.nih.gov/geo/) under Accession Numbers GSE12632, GSE24212, and GSE104491, respectively] may contribute to studies on other aspects on metabolism and metabolic regulation in this industrially relevant fungus.

Of five putative transporter genes that showed an over 30-fold higher transcript level in l-arabinose-limited chemostat cultures of \textit{P. chrysogenum} than in \(\alpha\)-glucose-limited cultures, only PcaraT was shown to encode an l-arabinose transporter that is functional in \textit{S. cerevisiae}. Whilst the low \(K_m\) of this transporter observed upon its expression in \textit{S. cerevisiae} is consistent with its upregulation in l-arabinose-limited cultures of \textit{P. chrysogenum}, this observation does not necessarily imply that PcaraT is the only or even the most important l-arabinose transporter active in these cultures. Problems in protein folding, plasma membrane (mis)targeting, post-translational modification and/or protein turnover [21, 68] may have affected expression of the other candidate genes. Indeed, in screening of cDNA libraries encoding putative heterologous transporters, typically only few of the candidate genes are found to enable transport of the substrate upon expression in \textit{S. cerevisiae} [69, 70].

Several studies have used gal2Δ strains of \textit{S. cerevisiae} to analyse transport kinetics of heterologous l-arabinose transporters (Table 6, Fig. 3). Two studies that estimated \(K_m\) and \(V_{\text{max}}\) of Gal2 upon its reintroduction in such a strain found different results (Table 6) [19, 26]. At l-arabinose concentrations of about 10 mmol L\(^{-1}\), these studies reported Gal2-mediated transport rates of 0.3 and 8.9 mmol (mg biomass)\(^{-1}\) min\(^{-1}\), respectively, as compared to a value of 2.5 mmol (mg
One of the previous studies [26] used a strain that also expressed a functional bacterial l-arabinose pathway, thereby raising the possibility that apparent uptake rates were enhanced by subsequent metabolism of l-arabinose. Moreover, in different studies, GAL2 was expressed from different promoters (pTDH3, pADH1 and pHXT7) and either high-copy-number (2µm) [19, 26] or low-copy-number centromeric (this study) expression plasmids. d-Glucose transport kinetics via

Table 6 Comparison of key characteristics of Gal2, PcAraT and heterologous l-arabinose transporters that were previously expressed in S. cerevisiae

| Protein | Origin | $K_m$, ARA [mM] | $V_{max}$, ARA [nmol (g biomass)$^{-1}$ min$^{-1}$] | GLC transport | XYL transport | Mechanism | References |
|---------|--------|-----------------|---------------------------------|---------------|--------------|-----------|------------|
| ScGal2  | S. cerevisiae | 335 ± 21.0 | 75 ± 5 | ✓ | ✓ | Facilitated diffusion | This study [19] |
| PcAraT  | P. chrysogenum | 0.13 ± 0.03 | 5.3 ± 0.2 | ✗ | ✗ | H$^+$ symport | This study |
| SsAraT  | Scheffersomyces stipitis | 3.8 ± 1.7 | 0.4 ± 0.1 | ✓ | ✗ | nd | [19] |
| AtStp2  | Arabidopsis thaliana | 4.5 ± 2.2 | 0.6 ± 0.1 | ✓ | ✗ | H$^+$ symport | [19] |
| KmAxt1  | Kluyveromyces marxianus | 263 ± 57 | 57 ± 6 | ✓ | ✗ | Facilitated diffusion | [26] |
| PgAxt1  | Pichia guilliermondii | 0.13 ± 0.04 | 18 ± 0.8 | ✓ | ✓ | H$^+$ symport | [26] |
| AmLat1  | Ambrosiozyma monospora | 0.03* | 0.2 ± 0.0 | ✗ | ✗ | nd | [70, 73] |
| AmLat2  | A. monospora | nd | 4 ± 0 | ✗ | ✗ | nd | [70, 73] |
| NcLat-1 | Neurospora crassa | 58 ± 4 | 194 ± 50 | ✓ | nd | H$^+$ symport | [30] |
| MtLat-1 | Myceliophthora thermophila | 29 ± 4 | 172 ± 6 | ✓ | nd | H$^+$ symport | [30] |

nd, not determined; ARA, l-arabinose; GLC, d-glucose; XYL, d-xylose. * $K_m$ of AmLat1 was determined as a GFP-fusion protein [73]

Fig. 3 Phylogenetic tree of S. cerevisiae Gal2, PcAraT and other heterologous l-arabinose transporters that have previously been functionally expressed in S. cerevisiae. Species names are added in two-letter code in front of protein names. Numbers are derived from a 500 times bootstrap iteration. Characteristics and literature references for each transporter are provided in Table 6. Accession numbers: ScGal2: P13181, PcAraT: CAP85508, SsAraT: A3LQ55-1, AtStp2: OAP13698, KmAxt1: GZ791039, PgAxt1: GZ791040, AmLat1: AY923868, AmLat2: AY923869, NcLat-1: EAA30346, MtLat-1: G2QFT5-1
Gal2 determined in this study \( K_m = 1.9 \text{ mmol L}^{-1}, \)
\( V_{\text{max}} = 26 \text{ mmol (mg biomass)}^{-1} \text{ min}^{-1} \) were similar to
previously reported values \([1.5 \text{ mmol L}^{-1} \text{ and } 27 \text{ mmol} \) (mg biomass)\(^{-1} \text{ min}^{-1} \)] [20].

\( L \)-Arabinose transport rates in \( L \)-arabinose-limited
chemostat cultures of both Gal2- and \( Pc\text{AraT} \)-dependent
strains were higher than the \( V_{\text{max}} \) values calculated from
transporter assays with radioactively labelled \( L \)-arabinose. A similar difference between transport
assays and rates of \( L \)-arabinose uptake in growing cultures
was reported by Knoshaug et al. [26]. These discrepancies suggest that either the transport assays did
not accurately reflect zero-trans-influx kinetics [71] or
that differences in experimental conditions and/or cell-
ular energy status between transport assays and chem-
ostat cultures influenced \( L \)-arabinose uptake. Assuming
that \( Pc\text{AraT} \) mediates symport of \( L \)-arabinose with a single proton, the \( L \)-arabinose consumption rate in
aerobic, \( L \)-arabinose-limited chemostat cultures of the
strain IMX1508 (\( Pc\text{AraT gal2Δ} \) (0.8 mmol (g bio-
mass))\(^{-1} \text{ h}^{-1} \); Table 5) would, under anaerobic condi-
tions, correspond to an ATP production rate of ca.
0.3 mmol ATP (g biomass))\(^{-1} \text{ h}^{-1} \). This rate of ATP pro-
duction is well below the reported ATP requirement
of anaerobic \( S. cervevisiae \) cultures for cellular mainte-
nance [ca. 1 mmol ATP (g biomass))\(^{-1} \text{ h}^{-1} \)] [72]. Con-
sistent with this observation, no growth on \( L \)-arabinose
as the sole carbon source was observed in anaerobic
shake flask cultures of the \( L \)-arabinose specialist strain
IMX869 strain (\( Pc\text{AraT gal2Δ} \) (data not shown).

Differences in experimental protocols for strain con-
struction and sugar uptake studies, as well as the differ-
ent kinetics observed in transport assays and growing
cultures, complicate quantitative comparisons between
different studies. Nevertheless, some important differ-
ences can be discerned between the heterologous \( L \)-ara-
inose transporters that have hitherto been expressed in
\( S. cervevisiae \) (Table 6, Fig. 3). Protein sequence align-
ment of \( Pc\text{AraT} \) and transporters that were previously
shown to mediate \( L \)-arabinose import in \( S. cervevisiae \) showed
that \( Pc\text{AraT} \) clusters with \( Ambrosiozyma mon-
ospora AmLat1 \) (Fig. 3). In terms of its low \( K_m \), \( Pc\text{AraT} \)
most closely resembled \( AmLat1 \) and the \( P. guilliermon-
dii PgAxt1 \) transporter. However, expression in \( S. cerve-
visiae \) of \( AmLat1 \) [70, 73] led to ~25-fold lower reported
\( V_{\text{max}} \) of \( L \)-arabinose uptake than found in the present
study for \( Pc\text{AraT} \). In contrast to \( Pc\text{AraT} \), \( PgAxt \) was able
to transport \( \pi \)-glucose, which might contribute to the
strong inhibition of the latter transporter by \( \pi \)-glucose
[26]. Although \( Pc\text{AraT} \) resembled \( A. thaliana \) Stp2 [19]
in being partially inhibited by \( \pi \)-glucose despite an ina-
bility to transport this sugar, \( Pc\text{AraT} \) enabled consump-
tion of \( L \)-arabinose in batch cultures containing 20 g L\(^{-1} \)
\( \pi \)-glucose.

In common with other high-affinity sugar transporters
in yeasts and fungi [26, 74], the observation that \( Pc\text{AraT} \) mediates \( L \)-arabinose-proton symport should be taken
into account in future strain designs, since simultaneous
activity of proton symport and facilitated diffusion, e.g.
via Gal2, may result in energy-consuming futile cycles
[8].

In the lignocellulosic hydrolysates now used in the
first industrial-scale plants for ‘second generation’
bioethanol production, \( L \)-arabinose generally rep-
resents between 2 and 3% of the total sugars [8]. At
the resulting low concentrations of \( L \)-arabinose in
the industrial processes, Gal2 operates far from sub-
strate saturation and is, moreover, strongly inhibited
by \( \pi \)-glucose. Based on its kinetic characteristics, as
analysed in transport assays and growing cultures,
\( Pc\text{AraT} \) represents an interesting candidate transporter
for evaluation of \( L \)-arabinose co-consumption under
industrial conditions. If the characteristics of \( Pc\text{AraT} \)
determined in the present study can be reproduced in
industrial strains and under industrial conditions, this
transporter can contribute to a timely and efficient con-
version of \( L \)-arabinose, and thereby to the overall pro-
cess economics.

**Conclusion**

Transcriptome analyses of \( L \)-arabinose-limited \( P. chry-
osogenum \) chemostat cultures proved valuable for iden-
tification of the high-affinity \( L \)-arabinose transporter
\( Pc\text{AraT} \). Functional expression and characterization in
\( S. cervevisiae \) revealed a high affinity and specificity of
this transporter for \( L \)-arabinose (\( K_m = 0.13 \text{ mmol L}^{-1} \)),
combined with a limited sensitivity to inhibition by
\( \pi \)-glucose and \( \pi \)-xylose, which are present at high con-
centrations in lignocellulosic hydrolysates. These char-
acteristics differentiate \( Pc\text{AraT} \) from the endogenous \( S.
cervevisiae \) transporter capable of \( L \)-arabinose transport
(Gal2) and qualify it as a potentially valuable additional
element in metabolic engineering strategies towards
efficient and complete conversion of \( L \)-arabinose pre-
seent in second-generation feedstocks for yeast-based
production of fuels and chemicals.
Additional files

Additional file 1. Primers used in this study.

Additional file 2. Differentially expressed genes in P. chrysogenum chemo-

ccultures over three different conditions (Δ-glucose, Δ-arabinose, and ethanol-limited). The table includes 540 genes that were differentially expressed amongst Δ-glucose, Δ-arabinose, and ethanol-limited chemo-
ccultures of P. chrysogenum (7.5 g L⁻¹, 7.5 g L⁻¹, 5.8 g L⁻¹, respectively, D = 0.03 h⁻¹, T = 30 °C). 137 transcripts exhibited a fold-change higher than three on Δ-arabinose and lower than three on ethanol relative to the Δ-glucose condition (the top 137 genes presented in the table). Shown are averages from triplicate (Δ-glucose) or duplicate chemostat cultures (Δ-arabinose, ethanol), the respective standard deviation and the ratio of gene expression levels in the presence of Δ-arabinose over Δ-glucose (Ara vs. Glc), as well as ethanol over Δ-glucose (EtOH vs. Glc).

Additional file 3. Effect of Δ-glucose (a) and Δ-xylose (b) on the specific rate of Δ-arabinose uptake by PcAraT (filled triangles) and Gal2 (filled squares). Uptake experiments were performed with 50 mmol L⁻¹ ¹⁴C-Δ-arabinose in the presence of increasing concentrations of Δ-glucose (a) or Δ-xylose (b). Symbols indicate uptake rates observed with the Hxt1-7 and Gal2 deletion strain S. cerevisiae DS68625-PcaraT (filled triangles) and DS68625-GAL2 (filled squares), expressing either PcaraT or Gal2, respectively. Data are derived from duplicate experiments and shown as the average ± mean deviation.

Additional file 4. Specific rate of Δ-arabinose uptake by PcAraT. Uptake experiments were performed with increasing concentrations of ¹³C-Δ-arabinose with the Hxt1-7 and Gal2 deletion strain S. cerevisiae DS68625-PcaraT expressing PcaraT on a centromeric plasmid. No ¹³C-Δ-glucose uptake was observed for this strain. Data are derived from duplicate experiments and shown as the average ± mean deviation.

Additional file 5. Specific rate of Δ-glucose uptake by Gal2. Uptake experiments were performed with increasing concentrations of ¹³C-Δ-glucose with the Hxt1-7 and Gal2 deletion strain S. cerevisiae DS68625-GAL2 expressing Gal2 on a centromeric plasmid. Data are derived from duplicate experiments and shown as the average ± mean deviation.

Additional file 6. Specific rate of Δ-arabinose uptake by Gal2. Uptake experiments were performed with increasing concentrations of ¹³C-Δ-arabinose with the Hxt1-7 and Gal2 deletion strain S. cerevisiae DS68625-GAL2 expressing Gal2 on a centromeric plasmid. Data are derived from duplicate experiments and shown as the average ± mean deviation.

Additional file 7. Impact of proton-gradient uncoupling on transport activity. Transport rates of ¹³C-Δ-arabinose of the Hxt1-7 and Gal2 deletion strains DS68625-PcaraT and DS68625-GAL2 expressing either PcaraT (DS68625-PcaraT, white bars) or Gal2 (DS68625-GAL2, grey bars) on a centromeric plasmid. Transport rates were determined in 200 μl synthetic medium at a ¹³C-Δ-arabinose concentration of 2 mmol L⁻¹ upon addition of either 0.5 μL water, 0.5 μL DSMO, or 10 μM CCCP (0.5 μL of a stock solution dissolved in 100% DSMO) (A). Panel (B) shows the uptake capacity in % relative to the control (H₂O). Data are derived from duplicate experiments and shown as the average ± mean deviation.

Abbreviations
SM: synthetic medium; SMA: synthetic medium with 20 g L⁻¹ Δ-arabinose; SMAG: synthetic medium with 20 g L⁻¹ Δ-arabinose and 20 g L⁻¹ Δ-glucose; SMEG: synthetic medium with 2% ethanol and 3% glycerol; SMUrea: synthetic medium with urea;YPD: yeast peptone medium with 20 g L⁻¹ Δ-glucose;YPΔ: yeast peptone medium with 2% ethanol; YPEG: yeast peptone medium with 2% ethanol and 3% glycerol; APA: Δ-arabinose; GLC: Δ-glucose;XYL: Δ-xylose; OD: optical density; ORF: open-reading frame; nd: not determined; PPP: pentose phosphate pathway.

Authors’ contributions
The experimental work including fermentation, strain construction and characterization was performed by JMB and MDV assisted by HWW and BC. JMB, MDV, and JG performed transcriptome data analysis. JMB, MDV, AJMD, AJAVM, PK, J-MGD, and JTP designed the study. JMB, MDV, and JTP wrote a draft version of manuscript. All authors commented upon the manuscript. All authors read and approved the final manuscript.

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Competing interests
Several of the authors are inventors on patent applications related to pentose fermentation by S. cerevisiae. DSM markets technology for biofuels production from lignocellulosic feedstocks, holds IP positions in this field and co-funded the research described in this publication.

Availability of data and materials
The datasets supporting the conclusions of this article are available at NCBI Genome Omnibus database (GEO) under Accession Numbers GSE12632, GSE24212, and GSE10449, respectively, in the GEO repository (https://www.ncbi.nlm.nih.gov/geo/).

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