Appl Microbiol Biotechnol (2014) 98:3569–3578
DOI 10.1007/s00253-013-5225-y

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Molecular cloning and characterization of two novel fructose-specific transporters from the osmotolerant and fructophilic yeast *Candida magnoliae* JH110

Dae-Hee Lee · Soo-Jung Kim · Jin-Ho Seo

Received: 18 July 2013 / Revised: 27 August 2013 / Accepted: 29 August 2013 / Published online: 19 September 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract Sugar transport is very critical in developing an efficient and rapid conversion process of a mixture of sugars by engineered microorganisms. By using expressed sequence tag data generated for the fructophilic yeast *Candida magnoliae* JH110, we identified two fructose-specific transporters, *CmFSY1* and *CmFFZ1*, which show high homology with known fructose transporters of other yeasts. The *CmFSY1* and *CmFFZ1* genes harbor no introns and encode proteins of 574 and 582 amino acids, respectively. Heterologous expression of the two fructose-specific transporter genes in a *Saccharomyces cerevisiae*, which is unable to utilize hexoses, revealed that both transporters are functionally expressed and specifically transport fructose. These results were further corroborated by kinetic analysis of the fructose transport that showed that *Cm*Fsy1p is a high-affinity fructose–proton symporter with low capacity (K_M=0.13± 0.01 mM, V_{max}=2.1±0.3 mmol h^{-1} $[gdw]^{-1}$) and that *Cm*Ffz1p is a low-affinity fructose-specific facilitator with high capacity (K_M=105±12 mM, V_{max}=8.6±0.7 mmol h^{-1} $[gdw]^{-1}$). These fructose-specific transporters can be used for improving fructose transport in engineered microorganisms for the production of biofuels and chemicals from fructose-containing feedstock.

Keywords *Candida magnoliae* JH110 · Fructose transporters · Biofuels · Fructose-rich feedstock

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-5225-y) contains supplementary material, which is available to authorized users.

D.-H. Lee
Biochemicals and Synthetic Biology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, South Korea

S.-J. Kim · J.-H. Seo (✉)
Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, South Korea
e-mail: jhseo94@snu.ac.kr

S.-J. Kim · J.-H. Seo
Center for Food and Bioconvergence, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-921, South Korea

Introduction

Sugar transporters move carbohydrate molecules across the cellular plasma membrane and conduct the first step of the carbohydrate metabolic pathway in all living organisms. However, only a few sugar transporters, including hexose and pentose transporters, have been explored in the context of metabolic pathway engineering (Lee et al. 2002; Young et al. 2012). Conventional microbial metabolic engineering approaches presume that intracellular enzymes or pathways restrain maximum production rates and, thus, depend on optimizing specific metabolic enzymes (Young et al. 2012). However, the sugar transport rate places upper limits on intracellular metabolic activities, especially in the exogenous sugar catabolism (Wlaschin and Hu 2007). This limitation is apparent in the production of biofuels and chemicals from lignocellulosic biomass (Ha et al. 2011; Young et al. 2010). Moreover, inefficient sugar transport impedes industrial scale production of biofuels from renewable resources (Alper and Stephanopoulos 2009; Lee et al. 2002). Thus, heterologous expression of active sugar transporters in engineered microorganisms would improve the production of renewable biofuels and chemicals in which other limiting factors have already optimized.

A major focus of heterologous transporter expression has been in the field of improving the utilization of pentoses such as xylose and arabinose because a common fermentative yeast, *Saccharomyces cerevisiae*, cannot readily metabolize

these five-carbon sugars (Hahn-Hägerdal et al. 2007; Hector et al. 2008; Jeffries and Jin 2004). A significant effort has been made to identify heterologous pentose-specific transporters for functional expression in engineered xylose-fermenting *S. cerevisiae* strains (Du et al. 2010; Leandro et al. 2006; Saloheimo et al. 2007; Young et al. 2011). These prior studies indicate that expression of a heterologous xylose transporter can improve the ability of *S. cerevisiae* to utilize xylose (Hector et al. 2008; Katahira et al. 2008; Leandro et al. 2009). However, little attention has been paid to the improvement of hexose utilization in yeasts via heterologous transporter expression. Fructose is a highly fermentable sugar that is widely distributed in nature and easily produced from a good agricultural source, inulin, that is naturally occurring as a storage carbohydrate in many plants such as Jerusalem artichoke, chicory, dahlia, and yacon (Chi et al. 2011). While enhancing the fructose uptake ability of yeast is a potentially major factor for efficient fermentation of fructose-rich feedstock or co-fermentation of glucose and fructose, no work has examined heterologous fructose transporter expression for improving fructose utilization yet. This might be because of the fact that most of the proteins that have been experimentally identified for mediating fructose transport in yeasts and fungi have been shown to prefer glucose to fructose (Leandro et al. 2009) and only a few proteins have been experimentally identified for enabling fructose-specific transport in yeasts. This limitation underscores the need to identify efficient fructose-specific transporters in yeasts.

A high-affinity fructose-specific symporter was first identified in *Saccharomyces pastorianus* (Goncalves et al. 2000) and *Saccharomyces bayanus* (Sousa et al. 2004). Later, other high-affinity fructose symporters were found in the aerobic milk yeast *Kluyveromyces lactis* (Diezemann and Boles 2003) and the gray mold fungus *Botrytis cinerea* (Doehlemann et al. 2005). Recently, a high-affinity fructose symporter was reported from the commercial wine yeast *S. cerevisiae* EC 1118 (Galeote et al. 2010). These transporters do not accept glucose as a substrate and mediate the active transport of fructose by a proton symport mechanism. In addition, three fructose-specific transporters were isolated and characterized from the fructophilic yeasts *Zygosaccharomyces bailii* (Pina et al. 2004) and *Zygosaccharomyces rouxii* (Leandro et al. 2011). Unlike most other yeasts, including the typical, glucophilic yeast *S. cerevisiae*, these fructophilic yeasts consume fructose faster than glucose. *Candida magnoliae* JH110 isolated from honeycomb is also a fructophilic yeast known as an erythritol producer (Kim et al. 2004; Koh et al. 2003). Using the fermentation kinetics of glucose and fructose, we have shown that *C. magnoliae* JH110 prefers fructose to glucose as carbon source (Yu et al. 2006). In addition, *C. magnoliae* JH110 is an industrially important yeast because it is able to grow over a wide range of pH and sugar concentrations and to produce various sugar alcohols, glycerol, erythritol, and mannitol from fructose; however, it cannot produce ethanol from hexose sugars (Yu et al. 2006).

Here, we report the identification and characterization of two fructose-specific transporters from the fructophilic yeast *C. magnoliae* JH110 using expressed sequence tag (EST) data. Heterologous expression of the transporters in a *S. cerevisiae* mutant lacking its own hexose transporters and kinetic analysis of the sugar transport revealed that one of the transporters is a high-affinity fructose–proton symporter with low capacity and the other one is a low-affinity fructose-specific facilitator with high capacity.

Materials and methods

Strains, plasmids, and culture conditions

C. magnoliae JH110 wild-type strain [Korean Culture Center of Microorganisms (KCCM) (formerly Korean Federation of Culture Collection) (KFCC))-10900] was used for the preparation of genomic DNA and maintained on solid yeast–peptone–sucrose medium (10 g/L yeast extract, 20 g/L peptone, and 300 g/L sucrose). All polymerase chain reaction (PCR) products intended for sequence analysis were cloned into the pGEM-T Easy vector (Promega) to facilitate DNA sequencing. *Escherichia coli* DH5α was used for propagation and amplification of plasmids. It was grown at 37 °C in Luria–Bertani medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with 100 μg/mL ampicillin. *S. cerevisiae* EBY.VW4000 (derived from the strain CEN.PK2-1C, *MAT*a *Δhxt1-17 Δgal2 Δstl1 Δagt1 Δmph2 Δmph3 leu2-3, 112 ura3-52 trp1-289 his3-Δ1 MAL2-8^{c} SUC2*) was used as a host strain for the functional characterization of two transporters from *C. magnoliae* JH110. Because this yeast lacks the ability to transport hexoses (Wieczorke et al. 1999), it was routinely grown in yeast–peptone–maltose medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L maltose) or on solid yeast synthetic complete (YSC) medium composed of 6.7 g/L yeast nitrogen base without amino acids, 20 g/L maltose, and Synthetic Complete Supplement Mixture (catalog number 4400–022; BIO101). All strains were grown with orbital shaking at 200 rpm. YSC medium supplemented with Complete Supplement Mixture without tryptophan (catalog number 4511-0x2; BIO101) was used to select and maintain yeast cells transformed with pRS424-derived plasmids.

Construction of cDNA library and EST

The cDNA library was constructed as part of a *C. magnoliae* JH110 EST sequencing project that contributed to the comprehensive characterization of gene expression when *C. magnoliae* JH110 was exposed to external osmotic stresses (unpublished data). The osmophilic yeast *C. magnoliae*

JH110 was grown at 30 °C in yeast–peptone medium (10 g/L yeast extract and 20 g/L peptone) supplemented with 300 g/L glucose, fructose, galactose, or sucrose, and then harvested by centrifugation at 20,000×*g* for 5 min at 4 °C. The collected pellet was washed once with sterile water, resuspended with RNAlater solution (Ambion), and stored at −80 °C. Total RNA was extracted from each cultivation using the hot phenol method (Köhrer and Domdey 1991), pooled, and used for mRNA isolation with the PolyATtract mRNA Isolation Kit (Promega). The cDNA library was synthesized with a cDNA Synthesis Kit (Stratagene) and Gigapack III Gold Packaging Cloning Kit (Stratagene) according to the manufacturer's instructions. The constructed library titer was 2.5×10^6 recombinant plaque-forming units and a total of 1,152 clones were randomly chosen for sequencing. To generate *C. magnoliae* JH110 EST sequences, all of the sequences longer than 100 bp were clustered by the CAP3 sequence assembly program (Huang and Madan 1999).

Isolation of two fructose-specific transporters

The size of the cDNA clones containing the putative *CmFSY1* and *CmFFZ1* sequences lacking the 5′-upstream region was 1,054 and 1,121 bp, respectively, when they were first isolated by random sequencing of clones from the cDNA library of *C. magnoliae* JH110. The deduced amino acid sequences of the partial *CmFSY1* and *CmFFZ1* cDNAs showed high homology to those of the previously reported hexose transporters of other yeasts and fungi. The unknown genomic DNA sequences in the 5′-upstream region of the partial *CmFSY1* and *CmFFZ1* genes were identified by genome walking, performed according to protocols of the manufacturer of the DNA Walking SpeedUp Kit (Seegene). For the upstream sequences, two PCR amplifications, a primary amplification followed by nested PCR, were carried out. The primers used for genome walking are shown in Table S1 (see the Supplemental Material). The complete nucleotide sequences of *CmFSY1* and *CmFFZ1* were obtained after the 5′-flanking regions of the partial *CmFSY1* and *CmFFZ1* genes were cloned, sequenced, and assembled. Genomic DNA of *C. magnoliae* JH110 was isolated with the DNeasy Blood & Tissue Kit (Qiagen), but cell lysis was performed by incubation at 30 °C for 90 min with Zymolyase (Sigma-Aldrich). All PCR reactions were conducted with the AccuPower HL PCR PreMix (Bioneer, South Korea) and DNA sequences were determined at the National Instrumentation Center for Environmental Management (NICEM, South Korea).

Sequence analysis

Searches for nucleotide and protein sequence similarities were conducted using the BLAST algorithm at the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequences were obtained using the web-based translation tool of the Expert Protein Analysis System (ExPASy, http://web.expasy.org/translate). The hydropathy profile of the transporters was analyzed with the Protein Hydrophobicity Plots (http://www.vivo.colostate.edu/molkit/hydropathy) (Kyte and Doolittle 1982). Multiple sequence alignment of the deduced amino acid sequences of *CmFSY1* and *CmFFZ1* was performed with the corresponding sequences from various organisms using the GeneDoc program (Nicholas et al. 1997). On the basis of these alignment results, a phylogenetic tree was constructed with the MEGA 5.1 software (Tamura et al. 2011) using the neighbor-joining method (Saitou and Nei 1987). A bootstrap analysis (Felsenstein 1985) was performed with 1,000 replicates to test the relative support for branches produced by the neighbor-joining method. All analyzed sequences of transporters were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank).

Plasmid and strain construction

Next, we constructed the expression plasmids pFSY1 and pFFZ1, which express *CmFSY1* and *CmFFZ1* fused with N-terminal hemagglutinin (HA) and C-terminal green fluorescence protein (GFP), respectively. For this, GFP was amplified using the pGFP plasmid (Clontech) with the primers GFP1 (CCGGAATTCATGAGTAAAGGAGAAGAACTTTTCAC, *Eco*RI site is underlined) and GFP2 (ACGCGTCGACCTATTTGTATAGTTCATCCATGCCAT, *Sal*I site is underlined) and inserted into the high-copy number plasmid pRS424, which is carrying the strong and constitutive glyceraldehyde 3-phosphate dehydrogenase promoter, and the cytochrome c oxidase terminator of *S. cerevisiae* (Mumberg et al. 1995), digested with *Eco*RI and *Sal*I restriction enzymes, resulting in plasmid p424GFP. The *CmFSY1* open reading frame was PCR-amplified from the *C. magnoliae* JH110 genomic DNA with the primers FSY1F (CGGAATTCATGTCTACCACTGACATTAAGCTTGA, *Eco*RI site is underlined) and FSY1R (GGGATCCCTATACAGAGCTCTTCACAGAGATTGTG, *Bam*HI site is underlined) and subsequently cloned into plasmid pMK486, which contains 3×HA epitopes at the 5′ end of the multiple cloning site, to generate the plasmid pHACmFSY1. The 3×HA-*CmFSY1* was obtained by PCR amplification from the pHACmFSY1 and then cloned into p424GFP, creating the plasmid pFSY1. For the construction of pFFZ1, *CmFFZ1* fused with an N-terminal 1×HA epitope was PCR-amplified with the primers HFFZ1F (GACTAGTA**TGTACCCATACGATGTTCCTGACT**ATGGTGAAGTTGGGTGCTTCTG, the *Spe*I site is underlined and the HA epitope is in bold) and HFFZ1R (GGAATTCTTAGAAGATCTCCACGTTCTTCTGC, the *Eco*RI site is underlined) and inserted into the p424GFP digested with the

restriction enzymes *Spe*I and *Eco*RI. Transformation of pFSY1 or pFFZ1 into *S. cerevisiae* EBY.VW4000 was performed using the lithium acetate method as described (Schiestl and Gietz 1989).

Subcellular localization analysis

To examine the expression of both *C. magnoliae* JH110 transporters in the *S. cerevisiae* EBY.VW4000 strain, protein immunoblot analysis was carried out using an HA antibody. Membrane fractions of the EBY.VW4000 strains expressing each transporter were prepared according to the procedure described previously (Krampe et al. 1998). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 8 % polyacrylamide) and electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was incubated in 5 % (*w*/*v*) skim milk solution in TBST buffer (20 mM Tris–HCl, 150 mM NaCl, 0.05 % [*v*/*v*] Tween-20) for 1 h at room temperature. The HA-probe (Santa Cruz Biotechnology), diluted 1/200 in TBST buffer, was incubated overnight with shaking. The blots were washed three times in TBST and incubated for 30 min at room temperature with an anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich). The reaction was visualized using the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich). A confocal laser scanning microscope (MRC-1024, Bio-Rad) was used to determine the subcellular localization of the GFP-fused transporters in transformed yeast cells. Exponentially growing cells were immobilized on microscope slides and the excitation and emission spectra of whole cell suspensions in 0.1 M potassium phosphate buffer, pH 6.5, were scanned. The spectra of cells expressing *HA-CmFSY1-GFP* or *HA-CmFFZ1-GFP* were normalized to cell density and corrected for background by subtraction of the spectra determined for cells harboring the empty plasmid pRS424 cultured under identical conditions. Emission spectra were collected between 500 and 550 nm, with excitation at 489 nm and excitation and emission slit width of 3 nm.

Sugar uptake and proton symport assays

For the drop-test assay, *S. cerevisiae* EBY.VW4000 transformed with pFSY1 or pFFZ1 was harvested in the exponential phase and resuspended in sterile water to an optical density at 600 nm of 1.0. Cells were serially diluted in 20-fold steps and 10 μL of each diluted solution was spotted onto a tryptophan-free YSC plate supplemented with 2 % (*w*/*v*) of maltose, glucose, fructose, mannose, galactose, xylose, arabinose, ribose, sorbitol, or mannitol and incubated at 30 °C for 2 days. For the determination of fructose-triggered proton symport, yeast cells were grown in YSC medium to an exponential phase, washed three times with sterile water, and resuspended in sterile water to an optical density at 600 nm of 10. The cell suspension was incubated with stirring at 30 °C and the extracellular pH was recorded. Fructose solutions were added to a final concentration of 5 mM.

Kinetic sugar transport studies

The initial uptake rate of D-[U-^{14}C]fructose was determined as described previously (Loureiro-Dias and Peinado 1984). In brief, yeast cells were grown to an optical density at 600 nm of 0.8, harvested, and washed with demineralized ice-cold water. Cells were resuspended in 100 mM Tris-citrate buffer, pH 5.0, and equilibrated at 28 °C for 10 min. The transport assay was started by adding labeled sugars (D-[U-^{14}C]fructose or D-[U-^{14}C]glucose) and stopped after 5 s by adding ice-cold demineralized water and filtering the suspension. The filter was washed with ice-cold demineralized water, after which the scintillation fluid was added. Incorporation of radioactivity was measured by standard scintillation counting. Kinetic parameters were determined from transport activity measurements with various concentrations of fructose or glucose.

Nucleotide sequence accession number

The nucleotide sequences of *CmFSY1* and *CmFFZ1* have been submitted to the GenBank database under accession numbers KC147727 and KC147728, respectively.

Results

Cloning of *CmFSY1* and *CmFFZ1*

The cDNA library of *C. magnoliae* JH110 was constructed and used for the generation of ESTs (unpublished data). Among the more than 1,000 randomly sequenced EST sequences, a homology search of the NCBI database revealed that two ESTs encoded partial amino acid sequences with substantial similarity to the fructose-specific transporters *FSY1* from *S. pastorianus* (Goncalves et al. 2000) and *FFZ1* from *Z. bailii* (Pina et al. 2004). On the basis of these cDNA sequences, a 1,054-bp (*CmFSY1*) or 1,121-bp (*CmFFZ1*) sequence containing parts of the coding regions of the putative fructose transporter genes of *C. magnoliae* JH110 was used for genome walking PCR, a technique with which a known sequence region is extended to its unidentified flanking regions (Hwang et al. 2003). Two full-length cDNA sequences of a 1,987-bp gene encoding *CmFSY1* and a 2,163-bp gene encoding *CmFFZ1* consisted of a single 1,725-bp open reading frame coding for a polypeptide of 574 amino acids with a calculated molecular weight of 63.27 kDa and 1,749-bp open reading frame coding for a protein of 582 amino acids with a predicted molecular mass of 65.20 kDa, respectively.

Comparison of the *CmFSY1* and *CmFFZ1* genomic sequences with cDNA fragments obtained with reverse transcription-PCR revealed that the coding region of the two putative fructose transporters harbors no intron.

Sequence analysis of *CmFSY1* and *CmFFZ1*

The deduced amino acid sequences of *CmFSY1* and *CmFFZ1* were used for a similarity search with reported hexose transporters of other yeasts and fungi. *Cm*Fsy1p shares 61 % primary amino acid sequence identity and 77 % similarity with a putative sugar transporter of *Debaryomyces hansenii* (XM_461227). *Cm*Ffz1p exhibited the highest identity to *Z. bailii* Ffz1p (CAD56485, 68 % identity) followed by that of recently isolated *Z. rouxii* Ffz1p (CAR31108, 67 % identity) and Ffz2p (CAR28354, 62 % identity). The core 11–12 transmembrane-spanning domains were identified in both *Cm*Fsy1p and *Cm*Ffz1p (Fig. 1a), which is a characteristic of all hexose transporters and other carriers in the major facilitator superfamily (Saier et al. 1999). To investigate the structural specificity of the two fructose transporters, a phylogenetic tree was constructed on the basis of the full-length amino acid sequences of sugar transporters from various yeasts and fungi (Fig. 1b). Three distinct clusters were formed: the Fsy1p cluster, including *Cm*Fsy1p and three functionally characterized high-affinity fructose symporters (Fsy1p from *S. pastorianus* and two Ffz1p proteins from *K. lactis* and *B. cinerea*), the Ffz1p cluster containing *Cm*Ffz1p and recently isolated and characterized fructose-specific transporters (Ffz1p and Ffz2p from *Z. rouxii* and Ffz1p from *Z. bailii*), and the Hxtp cluster, including the hexose facilitator proteins. The Ffz1p cluster, which includes *Cm*Ffz1p, is apparently far related to other known yeast hexose transporters covering other fructose transporters characterized so far (Fig. 1b).

Functional expression in *S. cerevisiae* EBY.VW4000

To examine the functionality of the *CmFSY1* and *CmFFZ1* genes, the two transporter genes were cloned into the yeast expression vector pRS424 harboring HA and GFP. The resulting constructs pFSY1 and pFFZ1, which have the *CmFSY1* and *CmFFZ1* genes fused with N-terminal HA and C-terminal GFP, respectively, were used to transform the yeast strain EBY.VW4000, which lacks 20 genes for hexose transporters but can grow on minimal medium with maltose as sole carbon source (Wieczorke et al. 1999). Expression of both *C. magnoliae* JH110 transporters in the EBY.VW 4000 strain was verified by western blot analysis using an HA

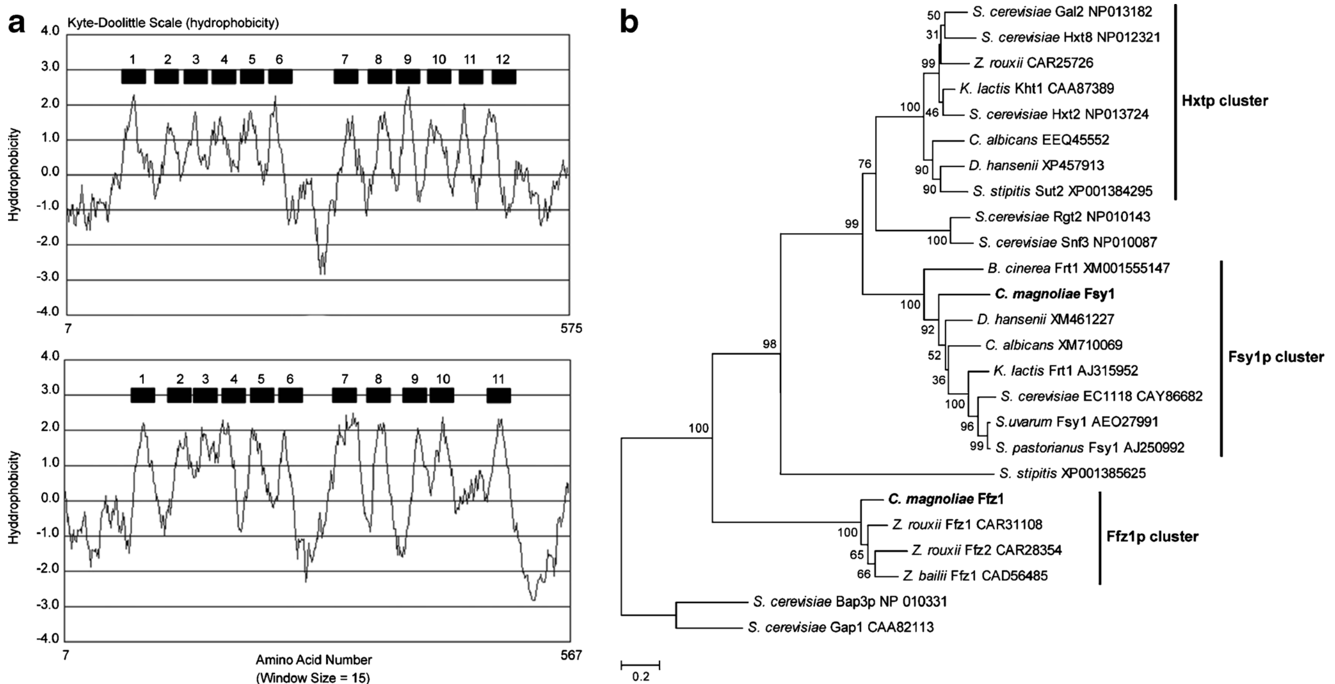

Fig. 1 Transporter topology prediction (**a**) and phylogenetic analysis (**b**) of two fructose-specific transporters of *C. magnoliae* JH110. **a** Topology prediction based on hydrophobicity was conducted using the Protein Hydrophobicity Plots with a window size of 15 (Kyte and Doolittle 1982). Hydrophobicity values are on the *y*-axis and the amino acid residue numbers are on the *x*-axis. The 12 predicted membrane-spanning domains (TM 1 to 12) are numbered. The *upper* and *lower plots* are for *Cm*Fsy1p and *Cm*Ffz1p, respectively. **b** A phylogenetic tree based on the similarity of deduced amino acid sequences was constructed with MEGA 5.1 software using the neighbor-joining method (applied to 1,000 bootstrap data sets) (Tamura et al. 2011). GenBank accession numbers are given after the species' designation. Protein acronyms are only noted for transporters that have been experimentally characterized. *Numbers at nodes* are bootstrap values based on 1,000 samplings

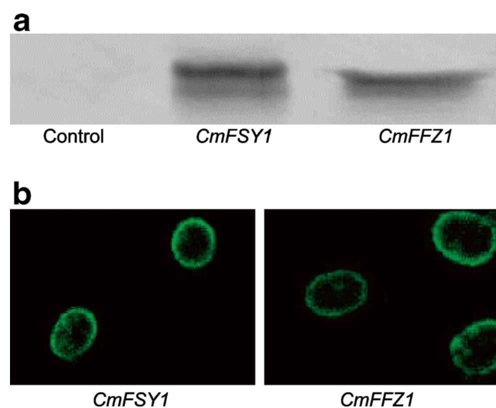

Fig. 2 Heterologous expression (**a**) and subcellular localization (**b**) of two fructose-specific transporters of *C. magnoliae* JH110. **a** Western blot detection was performed using a hemagglutinin (HA) antibody. Both *Cm*Fsy1p and *Cm*Ffz1p were expressed as fusion proteins with N-terminal HA and C-terminal green fluorescent protein (GFP) in *Saccharomyces cerevisiae* EBY.VW4000 lacking the ability to take up hexoses. Membrane fractions of EBY.VW4000 expressing the *CmFSY1* or *CmFFZ1* gene were prepared and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent immunoblot analysis. **b** Subcellular localization of both transporters in EBY.VW4000 cells was determined by confocal microscopy. Fluorescence of *Cm*Fsy1p or *Cm*Ffz1p fused with GFP was observed at the cell surface

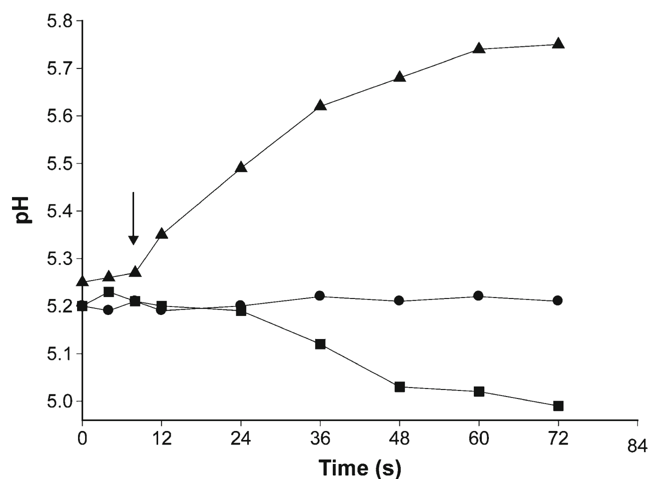

Fig. 3 Proton-coupled fructose symport mediated by *Cm*Fsy1p. Changes in the extracellular pH following addition of 5 mM fructose (*arrow*) to aqueous suspensions of yeast cells cultivated on maltose. *Saccharomyces cerevisiae* EBY.VW4000 cells harboring pFSY1 (*filled triangle*), pFFZ1 (*filled square*), or pRS424 (control, *filled circle*)

antibody (Fig. 2a). Incorrect localization of plasma membrane proteins overproduced in a heterologous context in the host cell has often been described (Wieczorke et al. 2003). In order to test this possibility, GFP was used as a reporter for the subcellular localization of heterologously expressed *CmFSY1* and *CmFFZ1* genes. Because fluorescence was clearly detected at the periphery of the transformed cells, both fusion proteins were correctly targeted to the plasma membrane of EBY.VW4000 (Fig. 2b). Moreover, the EBY.VW4000 strain harboring pFSY1 or pFFZ1 can grow on minimal medium with 2 % (*w*/*v*) fructose (specific growth rate—$0.12\pm0.01\ h^{-1}$ for pFSY1 and $0.15\pm0.02\ h^{-1}$ for pFFZ1), thereby confirming that both fusion proteins are functional fructose transporters located in the plasma membrane. Taken together, these results demonstrated that both fructose transporters are successfully expressed and correctly directed to the plasma membrane of *S. cerevisiae* EBY.VW4000.

Transport properties of *Cm*Fsy1p and *Cm*Ffz1p

Most hexose transporters from different organisms employ energy-independent facilitated diffusion or an active proton symport process (Leandro et al. 2009). To probe the involvement of protons in both fructose transporters, fructose-induced pH changes in aqueous cell suspensions were determined using EBY.VW4000 expressing *CmFSY1* or *CmFFZ1*. Proton influx resulting in external alkalization was observed when 5 mM fructose was added to aqueous suspensions of the fructose-grown cells expressing *CmFSY1* but not *CmFFZ1* (Fig. 3), as indicated by an increase in the extracellular pH triggered by the addition of 5 mM fructose. These results indicated that only *CmFSY1* encodes a proton-coupled fructose transporter. To further examine the transport properties of the fructose transporters, a drop-test assay was conducted on solid minimal medium with different carbon sources. As expected, all strains grew equally well on maltose, which is taken up by a specific transport system of the EBY.VW4000 strain (Fig. 4). Expression of the *CmFSY1* and *CmFFZ1* genes was able to restore growth on fructose, whereas no growth was observed on glucose (Fig. 4). In addition, growth on other hexoses (mannose and galactose), pentoses (xylose, ribose, and arabinose), or hexitols (sorbitol and mannitol) was also monitored. All strains showed no growth on any of these carbon sources (data not shown). Collectively, these results suggest that the *CmFSY1* and *CmFFZ1* genes encode a fructose-specific proton symporter and facilitative fructose transporter of *C. magnoliae* JH110, respectively.

Characterization of the kinetic parameters of *Cm*Fsy1p and *Cm*Ffz1p

The substrate specificity assessed by the earlier growth experiments was validated by measuring the initial rate of uptake of D-[U-^{14}C]fructose or D-[U-^{14}C]glucose in EBY.VW4000 cells expressing *CmFSY1* or *CmFFZ1*. No D-[U-^{14}C]glucose uptake could be observed (data not shown). A detailed analysis of their fructose affinities revealed that *Cm*Fsy1p is a high-affinity fructose-specific transporter with low capacity (K_M=0.13±0.01 mM, V_{max}=2.1±0.3 mmol h^{-1} $[gdw]^{-1}$), whereas *Cm*Ffz1p is a low-affinity fructose-specific transporter with high capacity (K_M=105±12 mM, V_{max}=8.6±0.7 mmol h^{-1} $[gdw]^{-1}$) (Fig. 5). The kinetic characteristics

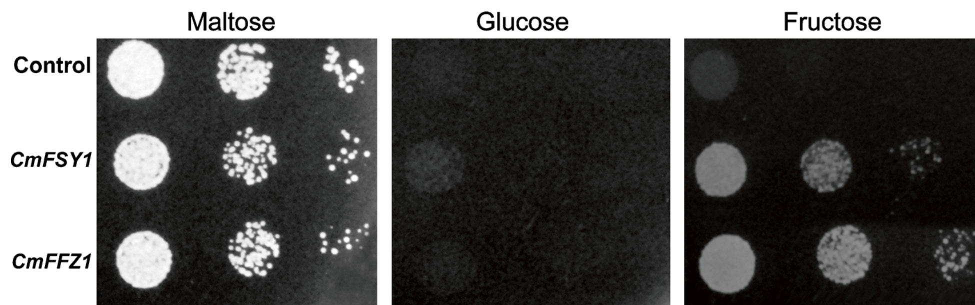

Fig. 4 Functional analysis of *Cm*Fsy1p and *Cm*Ffz1p expressed in *Saccharomyces cerevisiae* EBY.VW4000. Cells were serially diluted 20-fold (*left* to *right*), spotted on solid minimal medium supplemented with 2 % (*w*/*v*) maltose, glucose, or fructose and grown at 30 °C for 2 days. EBY.VW4000 cells transformed with pRS424 were used as a control strain

of fructose-specific transporters are summarized in Table 1. When comparing the kinetic parameters described for proton-coupled fructose symporters of other yeast strains, the affinity (K_M) and transport capacity (V_{max}) of *Cm*Fsy1p for fructose were comparable with those of *S. pastorianus* (Goncalves et al. 2000), *K. lactis* Fsy1p (Diezemann and Boles 2003), and *S. cerevisiae* EC1118 (Galeote et al. 2010), whereas the K_M value of *Cm*Fsy1p was much lower than the value determined in the *B. cinerea* fructose–proton symporter (Table 1). For *Cm*Ffz1p, the K_M value was similar to that of *Z. bailii* Ffz1p (Pina et al. 2004), but approximately four times lower than the value of *Z. rouxii* Ffz1p (Leandro et al. 2011) (Table 1).

Discussion

In this study, we investigated the first two functional fructose-specific transporters, *Cm*Fsy1p and *Cm*Ffz1p, identified in the fructophilic yeast *C. magnoliae* JH110, which differ in both their substrate affinity and transport capacity. (1) The *Cm*Fsy1 and *Cm*Ffz1 proteins are highly similar to functional fructose transporters from other yeasts. (2) Their expression in a *S. cerevisiae* hexose-transporter-deficient strain supports growth on fructose as sole carbon source. (3) Measurements of the initial fructose uptake rate and proton influx showed that *Cm*Fsy1p is a high-affinity fructose–proton symporter with low capacity and *Cm*Ffz1p is a low-affinity fructose-specific facilitator with high capacity.

Most yeast hexose transporters characterized at the molecular level are glucose transporters that do not differentiate between glucose and fructose as the substrate (Leandro et al. 2009). In *S. cerevisiae*, the affinity of hexose transporters for glucose is 5 to 10 times higher than that for fructose (Cirillo 1968; Kotyk 1967). Some exceptions to this are the high-affinity fructose-specific proton symporters first reported in the nonconventional yeasts *S. pastorianus* and *S. bayanus* (Cason et al. 1986; Goncalves et al. 2000; Sousa et al. 2004), which seem to be phylogenetically unrelated to the glucose transporters. In this study, *Cm*Fsy1p forms a Fsy1p cluster in the phylogenetic tree with four experimentally characterized proton–fructose symporters: Fsy1ps from *S. pastorianus* and *S. cerevisiae* EC1118 (Galeote et al. 2010; Goncalves et al. 2000), Frt1p from *K. lactis* (Diezemann and Boles 2003), and

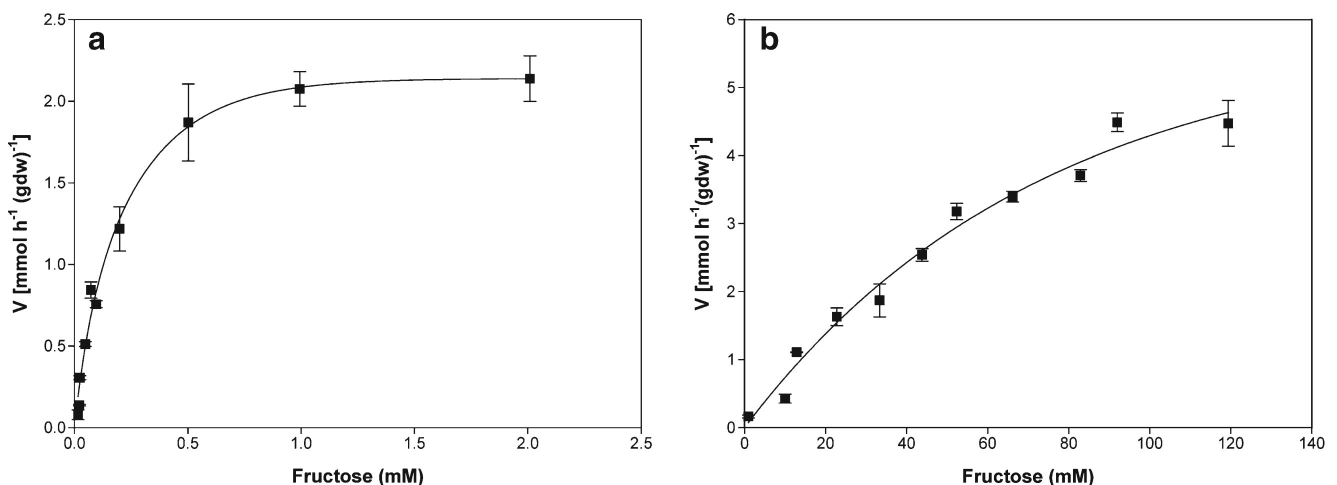

Fig. 5 Kinetic characteristics of fructose transport by yeast cells expressing *CmFSY1* (**a**) or *CmFFZ1* (**b**). Concentration-dependent transport activity of D-[U-^{14}C]fructose was measured and cells were incubated for 5 s with D-[U-^{14}C]fructose

Table 1 Kinetic parameters of fructose-specific transporters characterized in yeasts and fungi

Yeasts and fungi	Gene	Function	K_M (mM)	V_{max} (mmol h^{-1} $[gdw]^{-1}$)	Reference
Saccharomyces pastorianus	*FSY1*	H^+ symporter	0.16±0.02	3.8±0.2	Goncalves et al. (2000)
Saccharomyces cerevisiae EC1118	*FSY1*	H^+ symporter	0.24±0.04	0.93±0.08	Galeote et al. (2010)
Kluyveromyces lactis	*FRT1*	H^+ symporter	0.16±0.02	0.10±0.02[a]	Diezemann and Boles (2003)
Botrytis cinerea	*FRT1*	H^+ symporter	1.1	0.66[b]	Doehlemann et al. (2005)
Zygosaccharomyces rouxii	*FSY1*	H^+ symporter	0.45±0.07	0.57±0.02	Leandro et al. (2013)
Candida magnoliae JH110	*FSY1*	H^+ symporter	0.13±0.01	2.1±0.3	This study
Zygosaccharomyces bailii	*FFZ1*	Facilitator	80.4	3.3	Pina et al. (2004)
Zygosaccharomyces rouxii	*FFZ1*	Facilitator	424.2±163.1	12.7±3.3	Leandro et al. (2011)
Candida magnoliae JH110	*FFZ1*	Facilitator	105±12	8.6±0.4	This study

[a] The V_{max} value was recalculated from the original result (1.7±0.4 mmol min^{-1} $[mgdw]^{-1}$) to match the dimension (millimole per hour per gram dry weight)

[b] The V_{max} value was estimated from the Michaelis–Menten plot provided in the reference

BcFrt1 from *B. cinerea* (Doehlemann et al. 2005), suggesting that these energy-dependent active proton–fructose symporters have a common phylogenetic background (Fig. 1b). On the other hand, *Cm*Ffz1p is phylogenetically more distant from other described fungal hexose transporters. *Cm*Ffz1p and three *Zygosaccharomyces* fructose-specific transporters form a new fructose transport family, the Ffz1p cluster. These transporters are unrelated to other glucose or fructose transporters characterized so far, indicating that these fructose transporters using a facilitated diffusion system evolved differently from the other sugar transporter family members (Fig. 1b). Recently, two fructose-specific transporters were characterized in the fructophilic yeast *Z. rouxii* and classified into a new family of yeast sugar porters (Leandro et al. 2011). All members of this new fructose transporter family contain several highly conserved motifs that are different from those conserved in yeast hexose transporters (Pina et al. 2004). Those conserved motifs covering the 12 putative transmembrane domains are also observed in *Cm*Ffz1p (see Fig. S1 in the Supplemental Material). The other fungal fructose transporters belong to the large sugar porter family (Leandro et al. 2009) that transports both glucose and fructose by facilitated diffusion and has a higher affinity for glucose than fructose (e.g., *S. cerevisiae* hexose transporters). In addition to the fungal kingdom, one more type of eukaryotic fructose transporters, typified by the human facilitative fructose transporter GLUT5, was characterized (Kane et al. 1997). Whereas almost all eukaryotes have several copies of glucose transporters, some taxa seem to possess only one to approximately two copies of fructose-specific transporters. Among prokaryotes, only two fructose-specific ABC transporters with high affinity have been identified in the gram-negative bacterium *Sinorhizobium meliloti* (Lambert et al. 2001) and the gram-positive bacterium *Bifidobacterium longum* NCC 2705 (Wei et al. 2012).

When introduced in a *S. cerevisiae* mutant unable to transport fructose and glucose, both *C. magnoliae* fructose transporters were successfully expressed (Fig. 2a) and correctly targeted to the plasma membrane (Fig. 2b), thus supporting cell growth in the presence of fructose, but not glucose. The specific growth rate of the *S. cerevisiae* mutant expressing the *CmFSY1* gene on fructose was lower than that of the *CmFFZ1*-expressing mutant, indicating that *Cm*Fsy1p has a lower capacity for fructose than *Cm*Ffz1p. This was also corroborated by determining their kinetic parameters. The two transporters of *C. magnoliae* JH110 have the same substrate specificity but differ in their kinetic parameters. *Cm*Fsy1p was shown to be a high-affinity fructose-specific transporter with low capacity, whereas *Cm*Ffz1p was shown to be a low-affinity fructose-specific transporter with high capacity (Fig. 5). In general, the fructose–proton symporters characterized in yeasts show higher affinities and lower capacity than facilitated diffusion systems (Loureiro-Dias 1987; Peinado et al. 1989; Spencer-Martins and Van Uden 1985).

In contrast to *S. cerevisiae*, where hexoses are taken up only by facilitated diffusion, we have demonstrated that *C. magnoliae* JH110 harbors also an active fructose uptake system coupled with proton symport, *Cm*Fsy1p. To our knowledge, there is no previous report about the identification of two fructose-specific transporters from a single yeast. Even in the fructophilic yeasts *Z. bailii* and *Z. rouxii*, only fructose facilitators were identified and characterized. Although two fructose transporters (ZrFfz1p and ZrFfz2p) were recently identified in the osmotolerant and fructophilic *Z. rouxii* (Leandro et al. 2011), only ZrFfz1p is a fructose-specific facilitator; ZrFfz2p is a facilitator transporting glucose and fructose with similar affinity. In fructose-rich environments, the concentration gradient across the plasma membrane of *C. magnoliae* JH110 is sufficient to maintain an active catabolism because fructose crosses the membrane by facilitated diffusion without energy dissipation. Thus, the presence of a low-affinity and high-capacity fructose-specific transporter in *C. magnoliae* JH110 (*Cm*Ffz1p) is in agreement with the fact

that this yeast is isolated from high-fructose content honeycombs. In environments where fructose is present in low concentrations, *C. magnoliae* JH110 utilizes a proton motive force for fructose transport, i.e., through the high-affinity and low-capacity fructose-specific transporter *Cm*Fsy1p. Energy is then necessary to provide an adequate intracellular concentration of fructose to maintain the metabolism. This active transport mechanism and the high affinity of the transporter support the uptake of sugars even at very low concentrations. These characteristics are common in Crabtree-negative yeasts, including *C. magnoliae* JH110 (Van Urk et al. 1989).

In this study, we identified and characterized two fructose-specific transporters in *C. magnoliae* JH110. Their expression and engineering in industrial yeast strains could improve fructose uptake and, consequently, the performance of the engineered yeasts in the production of biofuels and chemicals from fructose-rich feedstock. More works are in progress to assess the effects of the expression of these fructose transporters on bioconversion of fructose-rich inulin to ethanol or erythritol in engineered *S. cerevisiae* or *C. magnoliae* JH110, respectively.

Acknowledgments This work was supported by the KRIBB Research Initiative Program and the Intelligent Synthetic Biology Center of Global Frontier Project funded by the Ministry of Education, Science, and Technology (2011–0031952). This work was also supported by the Advanced Biomass R&D Center (ABC) (2010–0029799).

References

Alper HS, Stephanopoulos G (2009) Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? Nat Rev Microbiol 7(10):715–723. doi:10.1038/nrmicro2186

Cason DT, Spencer-Martins I, van Uden N (1986) Transport of fructose by a proton symport in a brewing yeast. FEMS Microbiol Lett 36(2–3):307–309. doi:10.1111/j.1574-6968.1986.tb01715.x

Chi ZM, Zhang T, Cao TS, Liu XY, Cui W, Zhao CH (2011) Biotechnological potential of inulin for bioprocesses. Bioresour Technol 102(6):4295–4303. doi:10.1016/j.biortech.2010.12.086

Cirillo VP (1968) Relationship between sugar structure and competition for the sugar transport system in bakers' yeast. J Bacteriol 95(2):603–611

Diezemann A, Boles E (2003) Functional characterization of the Frt1 sugar transporter and of fructose uptake in *Kluyveromyces lactis*. Curr Genet 43(4):281–288. doi:10.1007/s00294-003-0392-5

Doehlemann G, Molitor F, Hahn M (2005) Molecular and functional characterization of a fructose specific transporter from the gray mold fungus *Botrytis cinerea*. Fungal Genet Biol 42(7):601–610. doi:10.1016/j.fgb.2005.03.001

Du J, Li S, Zhao H (2010) Discovery and characterization of novel D-xylose-specific transporters from *Neurospora crassa* and *Pichia stipitis*. Mol BioSyst 6(11):2150–2156. doi:10.1039/C0MB00007H

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39(4):783–791

Galeote V, Novo M, Salema-Oom M, Brion C, Valério E, Gonçalves P, Dequin S (2010) *FSY1*, a horizontally transferred gene in the *Saccharomyces cerevisiae* EC1118 wine yeast strain, encodes a high-affinity fructose/H^+ symporter. Microbiology 156(12):3754–3761. doi:10.1099/mic.0.041673-0

Goncalves P, Rodrigues de Sousa H, Spencer-Martins I (2000) *FSY1*, a novel gene encoding a specific fructose/H^+ symporter in the type strain of *Saccharomyces carlsbergensis*. J Bacteriol 182(19):5628–5630. doi:10.1128/jb.182.19.5628-5630.2000

Ha SJ, Galazka JM, Rin Kim S, Choi JH, Yang X, Seo JH, Louise Glass N, Cate JHD, Jin YS (2011) Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. Proc Natl Acad Sci U S A 108(2):504–509. doi:10.1073/pnas.1010456108

Hahn-Hägerdal B, Karhumaa K, Jeppsson M, Gorwa-Grauslund M (2007) Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. In: Olsson L (ed) Biofuels, vol 108, Advances in biochemical engineering/biotechnology. Springer, Berlin, pp 147–177

Hector R, Qureshi N, Hughes S, Cotta M (2008) Expression of a heterologous xylose transporter in a *Saccharomyces cerevisiae* strain engineered to utilize xylose improves aerobic xylose consumption. Appl Microbiol Biotechnol 80(4):675–684. doi:10.1007/s00253-008-1583-2

Huang X, Madan A (1999) CAP3: A DNA sequence assembly program. Genome Res 9(9):868–877. doi:10.1101/gr.9.9.868

Hwang IT, Kim YJ, Kim SH, Kwak CI, Gu YY, Chun JY (2003) Annealing control primer system for improving specificity of PCR amplification. Biotechniques 35(6):1180–1184

Jeffries TW, Jin YS (2004) Metabolic engineering for improved fermentation of pentoses by yeasts. Appl Microbiol Biotechnol 63(5):495–509. doi:10.1007/s00253-003-1450-0

Kane S, Seatter MJ, Gould GW (1997) Functional studies of human GLUT5: effect of pH on substrate selection and an analysis of substrate interactions. Biochem Biophys Res Commun 238(2):503–505. doi:10.1006/bbrc.1997.7204

Katahira S, Ito M, Takema H, Fujita Y, Tanino T, Tanaka T, Fukuda H, Kondo A (2008) Improvement of ethanol productivity during xylose and glucose co-fermentation by xylose-assimilating *S. cerevisiae* via expression of glucose transporter Sut1. Enzyme Microb Technol 43(2):115–119. doi:10.1016/j.enzmictec.2008.03.001

Kim HJ, Lee DY, Lee DH, Park YC, Kweon DH, Ryu YW, Seo JH (2004) Strategic proteome analysis of *Candida magnoliae* with an unsequenced genome. Proteomics 4(11):3588–3599. doi:10.1002/pmic.200400966

Koh ES, Lee TH, Lee DY, Kim HJ, Ryu YW, Seo JH (2003) Scale-up of erythritol production by an osmophilic mutant of *Candida magnoliae*. Biotechnol Lett 25(24):2103–2105. doi:10.1023/B:BILE.0000007076.64338.ce

Köhrer K, Domdey H (1991) Preparation of high molecular weight RNA. In: Christine Guthrie GRF (ed) Methods in enzymology, vol 194. Academic, Burlington, pp 398–405

Kotyk A (1967) Properties of the sugar carrier in baker's yeast. Folia Microbiol 12(2):121–131. doi:10.1007/BF02896872

Krampe S, Stamm O, Hollenberg CP, Boles E (1998) Catabolite inactivation of the high-affinity hexose transporters Hxt6 and Hxt7 of *Saccharomyces cerevisiae* occurs in the vacuole after internalization by endocytosis. FEBS Lett 441(3):343–347. doi:10.1016/s0014-5793(98)01583-x

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157(1):105–132. doi:10.1016/0022-2836(82)90515-0

Lambert A, Østerås M, Mandon K, Poggi MC, Le Rudulier D (2001) Fructose uptake in *Sinorhizobium meliloti* is mediated by a high-affinity ATP-binding cassette transport system. J Bacteriol 183(16):4709–4717. doi:10.1128/jb.183.16.4709-4717.2001

Leandro MJ, Fonseca C, Gonçalves P (2009) Hexose and pentose transport in ascomycetous yeasts: an overview. FEMS Yeast Res 9(4):511–525. doi:10.1111/j.1567-1364.2009.00509.x

Leandro MJ, Gonçalves P, Spencer-martins I (2006) Two glucose/xylose transporter genes from the yeast *Candida intermedia*: first molecular characterization of a yeast xylose-H^+ symporter. Biochem J 395(3): 543–549. doi:10.1042/BJ20051465

Leandro MJ, Sychrová H, Prista C, Loureiro-Dias MC (2011) The osmotolerant fructophilic yeast *Zygosaccharomyces rouxii* employs two plasma-membrane fructose uptake systems belonging to a new family of yeast sugar transporters. Microbiology 157(2):601–608. doi:10.1099/mic.0.044446-0

Leandro MJ, Sychrová H, Prista C, Loureiro-Dias MC (2013) *Zr*Fsy1, a high-affinity fructose/H^+ symporter from fructophilic yeast *Zygosaccharomyces rouxii*. PLoS ONE 8(7):e68165. doi:10.1371/journal.pone.0068165

Lee WJ, Kim MD, Ryu YW, Bisson L, Seo JH (2002) Kinetic studies on glucose and xylose transport in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 60(1–2):186–191. doi:10.1007/s00253-002-1085-6

Loureiro-Dias MC (1987) Glucose and polyol transport systems in *Candida intermedia* and their regulation. J Gen Microbiol 133(10): 2737–2742. doi:10.1099/00221287-133-10-2737

Loureiro-Dias MC, Peinado JM (1984) Transport of maltose in *Saccharomyces cerevisiae*. Effect of pH and potassium ions. Biochem J 222(2):293–298

Mumberg D, Müller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156(1):119–122. doi:10.1016/0378-1119(95)00037-7

Nicholas KB, Nicholas HB, Deerfield DW (1997) GeneDoc: analysis and visualization of genetic variation. EMBnew. News 4:14

Peinado JM, Cameira-Dos-Santos PJ, Loureiro-Días MC (1989) Regulation of glucose transport in *Candida utilis*. J Gen Microbiol 135(1): 195–201. doi:10.1099/00221287-135-1-195

Pina C, Goncalves P, Prista C, Loureiro-Dias MC (2004) Ffz1, a new transporter specific for fructose from *Zygosaccharomyces bailii*. Microbiology 150(7):2429–2433. doi:10.1099/mic.0.26979-0

Saier MHJ, Beatty JT, Goffeau A, Harley KT, Heijne WH, Huang SC, Jack DL, Jahn PS, Lew K, Liu J, Pao SS, Paulsen IT, Tseng TT, Virk PS (1999) The major facilitator superfamily. J Mol Microbiol Biotechnol 1(2):257–279

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406–425

Saloheimo A, Rauta J, Stasyk O, Sibirny A, Penttilä M, Ruohonen L (2007) Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases. Appl Microbiol Biotechnol 74(5):1041–1052. doi:10.1007/s00253-006-0747-1

Schiestl R, Gietz RD (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet 16(5–6):339–346. doi:10.1007/bf00340712

Sousa HRD, Spencer-Martins I, GonÁalves P (2004) Differential regulation by glucose and fructose of a gene encoding a specific fructose/H^+ symporter in *Saccharomyces sensu stricto* yeasts. Yeast 21(6): 519–530. doi:10.1002/yea.1118

Spencer-Martins I, Van Uden N (1985) Catabolite interconversion of glucose transport systems in the yeast *Candida wickerhamii*. BBA-Biomembranes 812(1):168–172. doi:10.1016/0005-2736(85)90535-8

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739. doi:10.1093/molbev/msr121

Van Urk H, Postma E, Scheffers WA, Van Dijken JP (1989) Glucose transport in Crabtree-positive and Crabtree-negative yeasts. J Gen Microbiol 135(9):2399–2406. doi:10.1099/00221287-135-9-2399

Wei X, Guo Y, Shao C, Sun Z, Zhurina D, Liu D, Liu W, Zou D, Jiang Z, Wang X, Zhao J, Shang W, Li X, Liao X, Huang L, Riedel CU, Yuan J (2012) Fructose uptake in *Bifidobacterium longum* NCC2705 is mediated by an ATP-binding cassette transporter. J Biol Chem 287(1):357–367. doi:10.1074/jbc.M111.266213

Wieczorke R, Dlugai S, Krampe S, Boles E (2003) Characterisation of mammalian GLUT glucose transporters in a heterologous yeast expression system. Cell Physiol Biochem 13(3):123–134. doi:10.1159/000071863

Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E (1999) Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett 464(3):123–128. doi:10.1016/S0014-5793(99)01698-1

Wlaschin KF, Hu WS (2007) Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. J Biotechnol 131(2):168–176. doi:10.1016/j.jbiotec.2007.06.006

Young E, Poucher A, Comer A, Bailey A, Alper H (2011) Functional survey for heterologous sugar transport proteins, using *Saccharomyces cerevisiae* as a host. Appl Environ Microbiol 77(10):3311–3319. doi:10.1128/aem.02651-10

Young EM, Comer AD, Huang H, Alper HS (2012) A molecular transporter engineering approach to improving xylose catabolism in *Saccharomyces cerevisiae*. Metab Eng 14(4):401–411. doi:10.1016/j.ymben.2012.03.004

Young EM, Lee SM, Alper HS (2010) Optimizing pentose utilization in yeast: the need for novel tools and approaches. Biotechnol Biofuels 3(1):24. doi:10.1186/1754-6834-3-24

Yu JH, Lee DH, Oh YJ, Han KC, Ryu YW, Seo JH (2006) Selective utilization of fructose to glucose by *Candida magnoliae*, an erythritol producer. Appl Biochem Biotechnol 131(1):870–879. doi:10.1385/ABAB:131:1:870