The osmotolerant fructophilic yeast\textit{Zygosaccharomyces rouxii} employs two plasma-membrane fructose uptake systems belonging to a new family of yeast sugar transporters

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Owing to its high resistance to weak-acid preservatives and extreme osmotolerance, \textit{Zygosaccharomyces rouxii} is one of the main spoilage yeasts of sweet foods and beverages. In contrast with \textit{Saccharomyces cerevisiae}, \textit{Z. rouxii} is a fructophilic yeast; it consumes fructose faster than glucose. So far, to our knowledge, no specific \textit{Z. rouxii} proteins responsible for this fructophilic behaviour have been characterized. We have identified two genes encoding putative fructose transporters in the \textit{Z. rouxii} CBS 732 genome. Heterologous expression of these two \textit{Z. rouxii} ORFs in a \textit{S. cerevisiae} strain lacking its own hexose transporters (\textit{hxt}-null) and subsequent kinetic analysis of sugar transport showed that both proteins are functionally expressed at the plasma membrane: ZrFz1 is a high-capacity fructose-specific facilitator ($K_m \sim 400 \text{ mM}$ and $V_{\text{max}} \sim 13 \text{ mmol h}^{-1} \text{ g}^{-1}$) and ZrFz2 is a facilitator transporting glucose and fructose with similar capacity and affinity ($K_m \sim 200 \text{ mM}$ and $V_{\text{max}} \sim 4 \text{ mmol h}^{-1} \text{ g}^{-1}$). These two proteins together with the \textit{Zygosaccharomyces bailii} Fz1 fructose-specific transporter belong to a new family of sugar transport systems mediating the uptake of hexoses via the facilitated diffusion mechanism, and are more homologous to drug/H\textsuperscript{+} antiporters (regarding their primary protein structure) than to other yeast sugar transporters of the Sugar Porter family.

\textbf{INTRODUCTION}

\textit{Zygosaccharomyces rouxii} as a spoilage yeast contaminates many food products such as sugar syrups, honey, fruit juices, sauces, carbonated soft drinks, salad dressings and ketchup. \textit{Z. rouxii} is also industrially used in the production of balsamic vinegar and in the alcoholic fermentation of some salted condiments, such as soy sauce and miso paste (Deák, 2007). In general, it can survive and even grow in the presence of weak-acid preservatives and high concentrations of salt or sugars. This unique ability is based on its high resistance to weak acids and extreme osmotolerance. \textit{Z. rouxii} strains are able to grow at 90\% (w/v) glucose, vigorously ferment hexose sugars (which can cause swelling or bursting in packs due to gas accumulation), grow at low pH, tolerate high temperatures and grow at high molar NaCl concentrations (Martorell \textit{et al.}, 2007).

\textit{Z. rouxii} is a fructophilic yeast, similar to its close relative \textit{Z. bailii}, which means that it consumes fructose faster than glucose, a behaviour opposite to that of \textit{Saccharomyces cerevisiae} (Emmerich & Radler, 1983). This fructophilic behaviour can be explained by the properties of glucose and fructose transporters and is based on three mechanisms. Fructose uptake is mediated by two different systems, a fructose-specific facilitator with high capacity and low affinity, and by a low-capacity and high-affinity facilitator that also transports glucose. In these two \textit{Zygosaccharomyces} species, fructose transport is thus prioritised over that of glucose, mainly at high sugar concentrations, since: (i) the transport system specific for fructose has a high capacity; (ii) fructose competes with glucose for the hexose transport system; and (iii) high concentrations of fructose inactivate the glucose facilitator (Sousa-Dias \textit{et al.}, 1996; S. Sousa-Dias and M. C. Loureiro-Dias, unpublished results).

The gene encoding the fructose-specific Fz1 transporter of \textit{Z. bailii} was isolated by functional complementation of an \textit{S. cerevisiae} strain lacking its own hexose transporters and thereby unable to grow on fructose or glucose as carbon sources. This protein only transports fructose ($K_m = 80.4 \text{ mM}$ and $V_{\text{max}} = 3.3 \text{ mmol h}^{-1} \text{ g}^{-1}$) and has a low level of sequence

\textit{Abbreviations:} DHA1, drug/H\textsuperscript{+} antiporter 1; SP, Sugar Porter.

Two supplementary figures and a supplementary table of primers are available with the online version of this paper.
similarly to previously characterized sugar transporters (Pina et al., 2004).

In most organisms, including yeasts, sugar transporters are crucial for supplying cells with energy and a source of carbon. Usually, yeasts have several transporters for the same sugar differing in their kinetic parameters and regulation (Leandro et al., 2009). The best studied so far has been the Hxt family of 20 hexose-transporter-related proteins that mainly transport glucose (Boles & Hollenberg, 1997).

Most sugar transporters from various organisms form the so-called Sugar Porter (SP) family that is the largest member of the Major Facilitator Superfamily. Generally, sugar transporters are single integral membrane proteins with two sets of six hydrophobic transmembrane-spanning α-helices and five sequence-conserved motifs, and they usually operate via energy-independent facilitated diffusion or active proton symport mechanisms. Most types of cells generally use facilitators, and sugar–proton symporters only operate when relatively low concentrations of sugar are available (Leandro et al., 2009).

In this paper, we describe the sequence analysis and cloning of two genes encoding fructose transporters in Z. rouxii CBS 732. Heterologous expression of the genes in an hxt-null S. cerevisiae strain and detailed kinetic characterization of their products revealed significant differences among the two transporters, one of which is fructose-specific and the other which has a broader substrate specificity for fructose and glucose.

**METHODS**

**Strains and growth media.** Z. rouxii CBS 732 was used for isolating the genes encoding putative fructose transporters. The S. cerevisiae BW31a strain (MATa leu2-3/122 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL Suc2 mal10 ena1-4:: HIS3 nh1:: LEU2; Kinclová-Zimmermannová et al., 2005) was used for constructing plasmids by homologous recombination. The S. cerevisiae CEN.PK2-1C (MATa leu2-3/112 ura3-52 trpl-289 his3A1 MAL2-86° Suc2; Entian & Köter, 2007) and hxt-null EBY.WV4000 (CEN.PK2-1C hxt17A hxt13A hxt15A hxt16A hxt14A hxt12A hxt9A hxt11A hxt10A hxt8A hxt514A hxt2A hxt367A gal2A slt1A aqt1A ydl247wa yfr160cA; Wieczorek et al., 1999) strains were used for expressing the putative fructose transporters. Yeast strains were grown in minimal YNB (yeast nitrogen base without amino acids containing the indicated carbon source and the required supplements) and richYPD (yeast extract, peptone, glucose) or YPM (yeast extract, peptone, maltose) media. Sugar concentrations are given as percentages (w/v). Escherichia coli XL1-Blue (Stratagene) was used as the host for plasmid amplification. E. coli transformants were grown in standard Luria-Bertani medium supplemented with ampicillin (100 μg ml⁻¹).

**Growth assays.** Yeast cells were grown at 30 °C and their growth was monitored either in drop tests on solid media or in liquid media using an ELx808 Absorbance Microplate Reader (BioTek Instruments) as described previously (Marésové & Sychrová, 2007).

**DNA manipulations.** These were performed according to standard protocols (Sambrook et al., 1989). Genomic DNA and plasmid DNA from yeast cells were isolated as described by Hoffman & Winston (1987). High-fidelity DNA polymerase Phusion F-530 (Finnzymes) was used to avoid mismatch base pairing during the synthesis of PCR products.

**Plasmid and strain constructions.** The plasmids used were YEp352 (empty multicopy plasmid; Hill et al., 1986) and pNHA1-985GFP (derived from pGRU1 plasmid, harbouring GFP-tagged NHA1 driven by its own promoter; Kinclová et al., 2001). Plasmids containing the ZYR001E10034g (ZrFFZ1) and ZYR001F20909g (ZrFFZ2) genes were constructed by homologous recombination in S. cerevisiae BW31a (Zaragoza, 2003). ZrFFZ1 (amplified by PCR with primers ZrFFZ1-GFP-F and ZrFFZ1-GFP-R) and ZrFFZ2 (amplified by PCR with primers ZrFFZ2-GFP-F and ZrFFZ2-GFP-R) genes were inserted behind the ScNHA1 promoter into the pNHA1-985GFP plasmid (previously digested with PvuI), resulting in the zrFFZ1-GFP and pZRF2-GFP plasmids. The ZrFFZ1 (amplified by PCR with primers ZrFFZ1-Y-F and ZrFFZ1-Y-R) and ZrFFZ2 (amplified by PCR with primers ZrFFZ2-Y-F and ZrFFZ2-Y-R) genes with their own promoters (828 and 869 bp, respectively) were inserted into the YEp352 plasmid (previously digested with BamHI), resulting in the pZRF1 and pZRF2 plasmids. The primers used for plasmid constructions are listed in Supplementary Table S1 (available with the online version of this paper). Both hxt-null EBY.WV4000 and CEN.PK2-1C S. cerevisiae strains were transformed with all four constructed plasmids and control YEp352 and pNHA1-985GFP plasmids.

**Fluorescence microscopy.** Exponentially grown cells were spotted onto microscope slides and observed with an Olympus AX70 fluorescent microscope. For GFP visualization, a U-MWB fluorescent cube was used with a 450–480 nm excitation filter and 515 nm barrier filter.

**Sugar transport assays.** Initial [U-¹⁴C]glucose and [U-¹³C]fructose uptake rates were measured as described previously (Loureiro-Dias & Peinado, 1984). Each experiment was performed at least twice. Cultures were harvested at OD₆₀₀–0.8, washed twice with ice-cold water and resuspended in water to a final concentration of approximately 50 mg (dry weight) ml⁻¹. In a 15 ml conical glass tube, 20 ml of this cell suspension was mixed with 20 ml 100 mM Tris/citrate buffer, pH 5, and incubated at 25 °C for 5 min. The transport assay was started by adding 10 ml 5 x D-[³¹⁴C]glucose or D-[¹³C]fructose, prepared by mixing labelled and non-labelled sugar to obtain a final fructose concentration ranging from 5 mM (with specific activity 2000 c.p.m. nmol⁻¹) to 350 mM (with specific activity 25 c.p.m. nmol⁻¹) and a glucose concentration ranging from 0.2 mM (with specific activity 5000 c.p.m. nmol⁻¹) to 200 mM (with specific activity 100 c.p.m. nmol⁻¹). The reaction was stopped after 5 s by adding 5 ml ice-cold demineralized water, and filtering the suspension immediately through a moist Whatman GF/C filter. The filter was washed with 5 ml ice-cold demineralized water and transferred to a scintillation vial with 5 ml scintillation fluid (Optiphase ‘HiSafe’ 2, Perkin Elmer). Radioactivity was measured in a Tri-Carb 1600 CA liquid scintillation analyser (Packard Instruments). Each assay was performed in triplicate. For each sugar concentration, a control tube was prepared by adding 5 ml ice-cold demineralized water to the cell suspension plus Tris/citrate buffer before the addition of D-[³¹⁴C] sugar (reaction time 0 s). Kinetic parameters were estimated using GraphPad Prism version 5.00 (Graphpad Software, http://www.graphpad.com) for Michaelis-Menten regression analysis. For inhibition assays, 20 μl of a solution of the sugar to be tested as an inhibitor (prepared in 100 mM Tris/citrate buffer, pH 5) was added to 10 μl of the labelled sugar in the glass tube and the reaction was started by adding 20 μl of the cell suspension.

Dry weight was determined by placing 100 μl cell suspension into pre-weighted aluminium foil cups (in triplicate) and drying in a 70 °C
oven for 24 h. The existence of H\(^+\) movements associated with initial hexose uptake was assessed with a pH meter when adding hexose pulses to unbuffered cell suspensions, as described by Loureiro-Dias & Peinado (1984).

**RESULTS**

**Identification of putative Z. rouxii fructose transporters**

Preliminary results from fructose transport measurements in Z. rouxii CBS 732\(^T\) suggested the existence of a low-affinity high-capacity transporter specific for fructose (S. Sousa-Dias and M. C. Loureiro-Dias, unpublished results), similar to the one already characterized in Z. bailii (Pina et al., 2004). We used the sequence of Z. bailii Ffz1 to search the Z. rouxii Génolevures genome database (Sherman et al., 2009) and identified two ORFs (without introns) encoding putative transporters highly similar to ZbFfz1p. We designated the ZYRO0E10054 (GenBank accession no. XM_002499318, Gene ID 8204919) and ZYRO0F02090 (GenBank accession no. XM_002497242, Gene ID 8205043) sequences as ZrFFZ1 and ZrZFFZ2, respectively. At the protein level, ZrFfz1p is 628 aa and shares 67% identity with ZbFfz1p, whereas ZrFfz2p is shorter (601 aa) and possesses 64% identity to ZbFfz1p. The two Z. rouxii Ffz proteins are 68% identical and probably contain 12 transmembrane hydrophobic domains [as predicted by the HMMTOP Server v. 2.0 (Tusnády & Simon, 2001)].

When compared with the protein sequences of known sugar transporters of other yeasts and fungi, the three *Zygosaccharomyces* proteins seem to form a protein subfamily apparently unrelated to other known and well-characterized yeast hexose transporters of the SP family (Fig. 1). This family includes glucose and fructose transporters with both passive and active transport mechanisms, e.g. *S. cerevisiae* Hxt sugar-facilitators and the fructose/H\(^+\) antiporter.

**Fig. 1.** Dendrogram based on primary protein sequence homology depicting the phylogenetic relationship between ZbFfz1, ZrFfz1, ZrFfz2 transporters and other fungal hexose and drug transporters. The DHA1 family is boxed. Phylogenetic analysis was performed using the MUSCLE web server (Edgar, 2004) for multiple alignments. For the construction of the phylogenetic tree, the PHYLIP, PROTDIST and NEIGHBOR programmes (Felsenstein, 2005) were used. The resulting tree was drawn in TreeView software (Page, 1996). Represented proteins (and corresponding GenBank accession nos) are: BcFrt1, *Botrytis cinerea* fructose/H\(^+\) symporter (AAU87358.1); CaHgt1, *Candida albicans* glucose transporter (CAA76406.1); CaHgt19, *C. albicans* putative glucose/myo-inositol transporter (EAK98868.1); CaMdr1, *C. albicans* (benomyl, cycloheximide, methotrexate, fluconazole)/H\(^+\) antiporter (P28873.1); CmCyrR, *C. maltosa* cycloheximide/H\(^+\) antiporter (P32071.1); KlFrt1, *K. lactis* fructose/H\(^+\) symporter (CAC79614.1); KlHgt1, *K. lactis* high affinity hexose transporter (AAC49461.1); KlRag1, *K. lactis* low affinity hexose transporter (XP_453656.1); ScFrt1, *S. cerevisiae* fructose transporter (P38124.1); ScHxt1, *S. cerevisiae* hexose transporter 1 (AAC49461.1); ScHxt2, *S. cerevisiae* hexose transporter 2 (AAA34701.1); ScHxt7, *S. cerevisiae* hexose transporter 7 (AAC49461.1); ScHxt19, *S. cerevisiae* polyamine/H\(^+\) antiporter (Q07824.1); SpaFsy1, *S. pastorianus* fructose/H\(^+\) symporter (CAR31108.1); ZbFfz1, *Z. bailii* fructose transporter (CAR31108.1); ZbFfz2, *Z. rouxii* fructose and glucose transporter (CAR28354.1).
symporter Fsy1 from *Saccharomyces pastorianus*. In our analysis, surprisingly, their high sequence similarity clusters the three *Zygosaccharomyces* transporters to the drug/H\(^+\) antiporter-1 (DHA1) family of the TC database (Saier et al., 2009), i.e. among transporters with different substrate specificities and transport mechanisms (DHA1 family is boxed in Fig. 1). Based on this similarity, the Génolevures consortium named these two *Z. rouxii* proteins according to the *S. cerevisiae* polyamine/H\(^+\) antiporter ScTpo1.

**Functional expression in *S. cerevisiae***

To characterize the two putative *Z. rouxii* fructose transporters, we constructed multicopy plasmids harbouring the corresponding ORFs, either driven by the weak and constitutive ScNHA1 promoter and tagged with the GFP sequence at their 3\(^\prime\) ends or containing the genes with their own promoters by homologous recombination in *S. cerevisiae* BW31a. The four constructed plasmids were retrieved, and their sequences were confirmed by restriction analysis and used to transform two *S. cerevisiae* strains, EBY.VW4000 and CEN.PK2-1C. As negative controls, we used the empty plasmid YEp352 and the pNHA1-985GFP plasmid harbouring GFP-tagged ScNHA1 driven by its own promoter. The EBY.VW4000 strain lacks its hexose transporters and as a result exhibits highly inhibited growth on media with glucose or fructose as a carbon source, whereas its parental strain CEN.PK2-1C has functional hexose transporters.

The expression of both genes from multicopy plasmids was not toxic to yeast cells, since the growth rate of BW31a, CEN.PK2-1C (on 2 % fructose) or EBY.VW4000 (on 2 % maltose) strains transformed with the constructs was about the same as the growth of the same strains transformed with the negative control plasmids (results not shown).

GFP tagging confirmed the predicted plasma-membrane localization of both ZrFfz proteins (Fig. 2), and the amount of tagged proteins in the plasma membrane was similar to that of the ScNha1 alkali-metal-cation/proton antiporter expressed in a similar way.

Initial drop-test assays showed that both GFP-tagged and non-tagged *Z. rouxii* proteins were functional fructose transporters. Their presence enabled the growth of transformed EBY.VW4000 cells on media with fructose as the only carbon source, whereas transformation with the empty vector or the expression of an unrelated transporter (Nha1 cation/H\(^+\) transporter) did not result in cell growth on fructose. These results also showed that both *Z. rouxii* promoters, used in pZRF1 and pZRF2, were fully functional in *S. cerevisiae* and that C-terminal GFP-tagging did not abolish fructose transport capacity (results not shown).

Detailed growth assays with various sugars as the carbon and energy source both on solid (Fig. 3) and in liquid (Fig. 4) media suggested differing substrate specificities for both transporters. ZrFfz1 only mediates the uptake of fructose, whereas ZrFfz2 has a broader substrate specificity and transports both fructose and glucose but not galactose (Fig. 3). EBY.VW4000 cells expressing both *Z. rouxii* transporters could also grow slowly on a sucrose medium, as they secrete invertase, hydrolysing sucrose in the medium to glucose and fructose (Fig. 3).

The cultivation of *hxt*-null *S. cerevisiae* strains expressing ZrFfz1p or ZrFfz2p from their own promoters in liquid media with various concentrations of fructose or glucose (Fig. 4) showed that these *Z. rouxii* transporters support a slow growth of cells in the presence of as little as 0.5 % fructose (and glucose in the case of ZrFfz2), and maximal growth is reached in the presence of 2 % sugar. Surprisingly, higher sugar concentrations (10 or 20 %) partially inhibited the growth rate (Fig. 4), and the growth of cells with ZrFfz2 was inhibited by 10 % glucose or fructose to the same level (not shown). For the strain expressing ZrFfz1p, no growth was detected at any of the four glucose concentrations used (shown only for 2 % glucose, Fig. 4).

![Fig. 2. Phase-contrast (left) and epifluorescence (right) images of *S. cerevisiae* *hxt*-null strain EBY.VW4000 transformed with multicopy plasmids (expressing genes driven by NHA1 promoter) pNHA1-985GFP (a), pZRF1-GFP (b) and pZRF2-GFP (c), grown on YNB medium with 2 % maltose.](image-url)
The ZrFFz1 and ZrFFz2 transporters were also expressed in the parental strain of EBY.VW4000, the CEN.PK2-1C strain, which has all of its own glucose and fructose transporters, to determine whether they would be able to improve growth at high glucose or fructose concentrations, but no significant increase in growth rates was observed (results not shown).

Characterization of kinetic parameters of ZrFFz1 and ZrFFz2

The substrate specificity estimated by the earlier growth experiments was verified by measuring the initial rate of uptake of various hexoses either directly or as the ability of a hexose to inhibit the uptake of fructose (or glucose) in hxt-null S. cerevisiae cells expressing ZrFFz transporters from their own promoters. Altogether, seven hexoses were tested, and the fructose specificity of ZrFFz1 and the ability of ZrFFz2 to transport both fructose and glucose were both confirmed.

A detailed characterization of their kinetic parameters revealed that ZrFFz1p was a high-capacity low-affinity system specialized in fructose uptake ($K_m$ fructose 424.2 ± 163.1 mM and $V_{max}$ fructose 12.7 ± 3.3 mmol h$^{-1}$ g$^{-1}$; Fig. 5). Fructose transport via ZrFFz1p was not inhibited by sorbose, mannose or 2-deoxyglucose (results not shown). On the other hand, ZrFFz2p transported fructose and glucose with similar affinity and capacity ($K_m$ fructose 204.7 ± 51.1 mM and $V_{max}$ fructose 4.51 ± 0.56 mmol h$^{-1}$ g$^{-1}$; $K_m$ glucose 195.7 ± 60.9 mM and $V_{max}$ glucose 3.12 ± 0.59 mmol h$^{-1}$ g$^{-1}$; Fig. 5), and transport of radioactive glucose and fructose by ZrFFz2p was not inhibited by galactose, sorbose, mannose or 2-deoxyglucose (results not shown).

The fact that the expression of ZrFFZ1 results in a much higher fructose transport capacity ($V_{max}$ almost three times higher) than that of ZrFFZ2 is most likely not due to a stronger promoter of ZrFFZ1, because when both genes were expressed from the ScNHA1 promoter, cells with ZrFFz1 had a higher fructose transport capacity than cells expressing ZrFFz2 (also about three times higher; results not shown). This higher transport capacity also explains the slightly faster growth of hxt-null cells expressing ZrFFZ1 when compared with those expressing ZrFFZ2 in fructose media, both from their own promoters (Figs 3 and 4; for example growth rates in 2% fructose media were 0.146 ± 0.002 h$^{-1}$ for the strain expressing ZrFFZ1 and 0.116 ± 0.001 h$^{-1}$ for the strain expressing ZrFFZ2) and from the ScNHA1 promoter (Fig. 3 and results not shown; growth rates in 2% fructose media were 0.108 ± 0.009 h$^{-1}$ for the strain expressing ZrFFZ1 and 0.092 ± 0.001 h$^{-1}$ for the strain expressing ZrFFZ2).

Uranyl cations were described as a competitive inhibitor of glucose transport in S. cerevisiae (Fuhrmann et al., 1992). To test whether they affect the uptake of fructose and glucose via Z. rouxii transporters, the sugar transport was measured in the presence of 0.05 and 0.5 mM uranyl nitrate (results not shown). For both transporters and both sugars, no inhibitory effect of uranyl on uptake rate was observed. This situation is similar to Z. bailii, where
facilitated diffusion mechanism.

To determine the mechanism of sugar transport via the two Z. rouxii transporters, whether it is a passive facilitated diffusion or active symport with protons, we monitored the changes in extracellular pH in an unbuffered cell suspension after the addition of fructose. As no alkalinization of the cell suspension was observed upon sugar addition (not shown) and since the affinity of both transporters to their substrates is quite low, we conclude that both ZrFfz1 and ZrFfz2 most probably operate via the facilitated diffusion mechanism.

**DISCUSSION**

In this work we have identified and characterized the first two plasma-membrane sugar transporters from the fructophilic yeast Z. rouxii that differ in both their substrate specificity and their transport capacity. One of them (ZrFfz1) is highly specific for fructose and has a higher transport capacity for fructose than the other (ZrFfz2), which, on the other hand, besides fructose also transports glucose with a similar affinity and velocity. Both transporters have a rather low affinity for their substrates (in hundreds of mmol l⁻¹) and transport them via the facilitated diffusion mechanism.

ZrFfz1 and ZrFfz2 transporters are phylogenetically distant from other known fungal sugar transporters with the exception of Z. bailii Ffz1 fructose-specific transporter. The three Zypagosaccharomyces fructose transporters form a new sugar transport family that is not related to other fructose transporters characterized so far (Fig. 1). The other fungal fructose transporters belong to the large SP family (Leandro et al., 2009) and can be divided into two subfamilies according to the transport mechanism they use. Most of them transport both glucose and fructose by facilitated diffusion and have a higher affinity for glucose than for fructose (e.g. S. cerevisiae Hxt transporters). The other subfamily uses an active H⁺-symport mechanism, is fructose-specific and is typified by, for example, Fsy1 from S. pastorianus (Gonçalves et al., 2000), Fmr1 from Kluyveromyces lactis (Diezemann & Boles, 2003) or BcFrt1 from the grey mould fungus Botrytis cinerea (Doehlemann et al., 2005).

As far as we know, the three Zypagosaccharomyces fructose transporters are the first example of a yeast sugar-transporter subfamily unrelated to the SP family. Surprisingly, the sequences of Z. rouxii Ffz transporters, consisting of 12 transmembrane-spanning domains, seem to be related to the DHA1 family of active drug/H⁺ antiporters (Fig. 1). Therefore, the Ffz proteins form a new and unique family of yeast sugar transporters, indicating that proteins with a substantial degree of homology can operate by differing mechanisms and have very different substrates. Performing a **BLASTP** search in the NCBI protein database for proteins similar to the Ffz transporters revealed the existence of several putative proteins with a high degree of homology to the Ffz proteins (60–64% identity), belonging to some Aspergillus species (such as Aspergillus oryzae and Aspergillus nidulans) and several fungal plant pathogens (such as Gibberella zeae and Botrytis cinerea), which could indicate that they all belong to a new family of hexose transporters, more related at the sequence level to drug/H⁺ antiporters than to known fungal hexose transporters (Supplementary Fig. S1, available with the online version of this paper). In all these members of the new sugar-transporter family, several highly conserved sequence motifs could be found such as LGFLXFN in transmembrane segment (TMS) 2; LAPFCELXGR between TMS 2 and 3; GTITVGGTFTXD at the end of TMS 4; AIXGTXXAPI AGF in TMS 5; ETRG at the end of TMS 6 (in this region, the conserved motif of members of the SP family is PESPRXL); FLFSLVIIXTF at the end of TMS 7; and EGGXGPIYYXL in TMS 8. These motifs differ from those found conserved in the yeast hexose transporters of the SP family (Leandro et al., 2009) and are not conserved in yeast drug transporters of the DHA1 family (Supplementary Fig. S2, available with the online version of this paper).

Also, the fact that all three Zypagosaccharomyces fructose transporters are insensitive to uranyl cations and that ZrFfz2 transports glucose but not 2-deoxyglucose indicate that these proteins differ from other yeast hexose transporters. When expressed in S. cerevisiae mutants unable to transport fructose and glucose, both Z. rouxii transporters mainly support cell growth in the presence of 2% sugar. The delayed and slow growth observed in liquid media with 0.5% sugar suggests that these transporters have a rather low affinity for their substrates, which was also confirmed by estimating their kinetic parameters. The growth inhibition observed in the presence of higher sugar concentrations (over 10%) could be due to either the toxic

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**Fig. 5.** Initial uptake of fructose (a) and glucose (b) in S. cerevisiae hxt-null EBY.VW4000 cells grown in YNB medium with 2% fructose and expressing ZrFfz1p (a) or ZrFfz2p (b) (genes driven by their own promoters). Values are means of at least two independent experiments.
effect of surplus sugars inside the cells or higher osmotic pressure of the media.

As mentioned above, the two transporters differ in their substrate specificity (ZrFfz1 is fructose-specific whereas ZrFfz2 transports both fructose and glucose) and kinetic parameters. When comparing the kinetic parameters previously determined for fructose transport directly in Z. rouxii CBS 732 cells (K_m = 284.54 ± 62.84 mM and V_max = 17.48 ± 2.45 mmol h^{-1} g^{-1}; S. Sousa-Dias and M. Loureiro-Dias, unpublished results) with those determined in this work for ZrFfz1 and ZrFfz2, expressed in S. cerevisiae, ZrFfz1 (V_max = 12.7 mmol h^{-1} g^{-1}) seems to be the major system responsible for the high fructose transport capacity of Z. rouxii. To confirm this hypothesis, we plan to construct and characterize Z. rouxii mutants lacking one or both of the Ffz proteins, together with analysing the regulation of their expression. Z. rouxii strains are generally isolated from high sugar environments (Martorell et al., 2007) where sugar can efficiently enter the cells by facilitated diffusion (as in the case of ZrFfz1 and ZrFfz2) without the need to expend energy in active transport mechanisms. Whether there is another active fructose transporter in Z. rouxii used when the fructose concentrations are low remains an open question that we plan to address in the future.

Although the preliminary results of the expression of ZrFFZ genes in the CEN.PK2-1C strain that still has all of its functional hexose transporters (parental strain of hxt-null EBY.VW4000 strain) did not show a significant growth phenotype, it would be interesting to monitor the sugar consumption profiles and growth rates of S. cerevisiae strains expressing ZrFFZ genes as well as their own hexose transporters (with lower fructose transport capacity) in media with glucose and fructose to see if the expression of Z. rouxii genes can improve fructose consumption by S. cerevisiae cells. We hope that the identification and characterization of these Z. rouxii-specific fructose transporters and their expression in industrial S. cerevisiae strains could improve fructose uptake and consequently S. cerevisiae performance at high sugar concentrations preventing stuck fermentations and reducing contamination risks.

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