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Characterization of hexose transporters in *Yarrowia lipolytica* reveals new groups of Sugar Porters involved in yeast growth

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ABSTRACT

Sugar assimilation has been intensively studied in the model yeast *S. cerevisiae*, and for two decades, it has been clear that the homologous *HXT* genes, which encode a set of hexose transporters, play a central role in this process. However, in the yeast *Yarrowia lipolytica*, which is well-known for its biotechnological applications, sugar assimilation is only poorly understood, even though this yeast exhibits peculiar intra-strain differences in fructose uptake: some strains (e.g., W29) are known to be slow-growing in fructose while others (e.g., H222) grow rapidly under the same conditions. Here, we retrieved 24 proteins of the Sugar Porter family from these two strains, and determined that at least six of these proteins can function as hexose transporters in the heterologous host *Saccharomyces cerevisiae* EBY.VW4000. Transcriptional studies and deletion analysis in *Y. lipolytica* indicated that two genes, *YHT1* and *YHT4*, are probably the main players in both strains, with a similar role in the uptake of glucose, fructose, and mannose at various concentrations. The other four genes appear to constitute a set of 'reservoir' hexose transporters with an as-yet unclear physiological role. Furthermore, through examining Sugar Porters of the entire *Yarrowia* clade, we show that they constitute a dynamic family, within which hexose transport genes have been duplicated and lost several times. Our phylogenetic analyses support the existence of at least three distinct evolutionary groups of transporters which allow yeasts to grow on hexoses. In addition to the well-known and widespread Hxt-type transporters (which are not essential in *Y. lipolytica*), we highlight a second group of transporters, represented by Yht1, which are phylogenetically related to sensors that play a regulatory role in *S. cerevisiae*, and a third group, represented by Yht4, previously thought to contain only high-affinity glucose transporters related to Hgt1of *Kluyveromyces lactis*.

1. Introduction

Hexoses, particularly glucose and fructose, are readily assimilated by numerous microorganisms. These sugars are naturally present in vegetables and fruits, in which they are conveniently used by humans for food or beverage fermentation, as well as abundantly stored in the form of various polysaccharides, which serve as either carbon storage (e.g., starch, inulin, sucrose, galactomanan gums) or as the structural fibers of plants (e.g., cellulose, insoluble fructans). These hexoses attract much attention for their ability, along with that of the pentose fraction of lignocelluloses, to provide cheap sources of carbon and energy for biotechnological microorganisms.

As a first step in their assimilation, the sugars must be transported across the cytoplasmic membrane into the cell. This process has been extensively studied in the model yeast *Saccharomyces cerevisiae*, in which hexose transporters were found in a single group of homologous proteins encoded by *HXT* genes (up to 18 proteins, including Gal2). The expansion of the number of *HXT* genes may possibly have been related to the adaptation of *S. cerevisiae* to aerobic fermentation (Lin and Li, 2011; Wieczorke et al., 1999). However, the deletion of only seven of these transporters (hxt1-7) results in a lack or severe reduction (depending on the yeast strain involved) of growth on glucose-based media (Wieczorke et al., 1999). In *S. cerevisiae*, the regulatory network of the Hxt transporters is complex and includes the so-called hexose sensors, which are similar to the Hxt membrane proteins but

Abbreviations: aa, amino acids; SP, Sugar Porter.

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lack their transport function (Busti et al., 2010). These sensors are responsible for induction of the transporter genes at the appropriate glucose concentration (Ozcan and Johnston, 1999). Both the Hxt transporters and the hexose sensors belong to the Sugar Porter, or SP, protein family (TC 2.A.1.1) as described in the Transport Classification Database (http://www.tcdb.org/).

In addition to transporting glucose via facilitated diffusion, many of the Hxt proteins are also able to transport fructose using the same mechanism. However, some *S. cerevisiae* strains, such as EC1118, also contain a high-affinity fructose/H^+ symporter (Fsy1), which probably originated from another organism (Galeote et al., 2010). This protein is distantly related to Hxt but belongs to the same SP family. In addition, high-capacity, low-affinity transporters for fructose (Ffz1 and Ffz2) were recently discovered in the fructophilic yeasts *Zygosaccharomyces rouxii* and *Z. bailii* (Leandro et al., 2011; Pina et al., 2004). These transporters belong to the DHA1 protein family (TC 2.A.1.2), which is known to comprise mainly drug/H^+ antiporters.

Like *S. cerevisiae*, *Yarrowia lipolytica* belongs to the subphylum Saccharomycotina, but this yeast diverged long before the emergence of the Saccharomycetaceae. *Y. lipolytica* has high potential for industrial applications due to its ability to accumulate lipids and to be used for synthesis of aromas, organic acids, polyols, or emulsifiers. Its natural assimilation of sugars is restricted to a rather narrow spectrum, including some polyols and the hexoses glucose, mannose, and fructose (Kurtzman, 2011; Michely et al., 2013). Metabolic pathways that are specific to other sugars, such as galactose, xylose, or cellobiose, may need to somehow be activated to sustain growth (Lazar et al., 2015; Ryu et al., 2015). Unexpectedly, our recent research revealed inter-strain variation in growth in fructose medium: the French strain W29 grows slowly in medium-to-high concentrations of fructose while the German strain H222 grows rapidly. One of the factors contributing to this inter-strain variation is diversity in hexokinase activity, which revealed sugar phosphorylation to be a limiting step for fructose assimilation in W29 and its obese derivatives (Lazar et al., 2014). Both strains H222 and W29, however, demonstrate a strict preference for glucose over fructose when both sugars are present (Lazar et al., 2013; Moeller et al., 2012). To date, the molecular mechanisms behind this behavior are unknown.

Experimental data on hexose transporters in *Y. lipolytica* remain scarce, and no fructose transporter has been clearly identified. Early *in silico* genome mining of the E150 (CLIB22) strain did not clearly indicate any hexose transporters because all candidates were considered too divergent from *S. cerevisiae* Hxt transporters (De Hertogh et al., 2006). The phylogenetic analyses of Palma et al. later revealed the existence of four gene products that are related to *S. cerevisiae* Hxt transporters or to a high-affinity glucose transporter of *Kluyveromyces lactis* (Palma et al., 2007), and two that are closer to glucose (hexose) sensors (Palma et al., 2009). However, only one glucose transporter has been experimentally identified (YALI0C06424), which reportedly exhibited no detectable fructose transport activity in complementation tests using a *Saccharomyces* heterologous host (Young et al., 2011). As the next step in deciphering sugar assimilation in *Y. lipolytica*, we decided to characterize its hexose transporters by investigating two different strains of this species, W29 and H222. We individually screened members of the Sugar Porter family for their hexose transport ability using an appropriate heterologous host and identified physiologically active genes by disruption and transcription analysis in *Y. lipolytica*. We observed that these hexose transporters belong to distinct phylogenetic groups. Finally, we investigated their evolutionary history in several species related to *Y. lipolytica*.

2. Materials and methods

2.1. Strains and general cultivation

Y. lipolytica was routinely grown on YP medium that contained either 1% (w/v) glucose (YPD) or glycerol (YPG). Minimal medium (YNB) consisted of 1.7 g/L yeast nitrogen base (without amino acids and ammonium sulfate, Difco ref. 233520), 5 g/L NH_4Cl, and 50 mM phosphate buffer pH 6.8; this was supplemented with uracil (0.1 g/L) or leucine (0.2 g/L) for auxotrophic strains. A carbon source was added as indicated in the text. The natural isolates W29 (Barth and Gaillardin, 1996) and H222 (Mumberg et al., 1995) were used as sources of transporter genes. Derivatives of strain PO1d (*MatA*, *leu2-270*, *ura3-302*, *xpr2-322*, CLIB 139) (Barth and Gaillardin, 1996), which is itself derived from W29, were used for the construction of strains with transport mutations. An auxotrophic strain of H222 (*MATA ura3-41*)(Mauersberger et al., 2001) served as a recipient for the invertase expression cassette, whereas the previously constructed JMY2531 (already carrying invertase expression cassettes) (Lazar et al., 2013) was used for the W29 background.

S. cerevisiae strain EBY.VW4000 (CEN.PK2-1C Δ*hxt1-17* Δ*gal2* Δ*stl1* Δ*agt1* Δ*mph2* Δ*mph3*) (Wieczorke et al., 1999), kindly provided by Prof. E. Boles, was used for complementation tests. Plasmid-containing derivatives of this strain were grown in minimal YNBs medium, consisting of 6.5 g/L yeast nitrogen base and 10 g/L $(NH_4)_2SO_4$, supplemented with histidine (45 mg/L), leucine (180 mg/L), and tryptophan (27 mg/L), and with 20 g/L maltose as a routine carbon source. Alternative carbon sources for growth tests are indicated in the text as appropriate.

Both *Y. lipolytica* and *S. cerevisiae* were cultivated at 28 °C. Flask cultures were performed with 170 rpm agitation in 250-mL Erlenmeyer flasks that were filled with 50 mL of the appropriate medium. *E. coli* Mach1T1 (Invitrogen) was used as a host strain for gene cloning and was cultivated in standard LB medium with 0.05 mg/L kanamycin for the selection of recombinant plasmids.

2.2. Identification and phylogeny of SP genes in the Y. lipolytica strains and in the Yarrowia clade

Two types of genome data sets were used in this study, both obtained in our laboratory at INRA. Genomes of species of the *Yarrowia* clade, i.e. *Y. yakushimensis* CBS10253, *C. galli* CBS9722, *Y. phangngensis* CBS10407, *C. alimentaria* CBS10151, and *C. hispaniensis* CBS9996, were annotated and manually curated as described (Meunchan et al., 2015). Scaffolds for *Yarrowia lipolytica* strains H222 and W29 were automatically annotated using the manually curated E150 genome as a source (Devillers, Brunel, Morin, Neuvéglise, unpublished results). In both cases, pseudogenes were annotated. Homologues of *Y. lipolytica* E150 SP genes were found by BLASTp (Altschul et al., 1990) with a cutoff E-value of $1e^{-10}$. Identified SP genes in the clade species have been deposited in the European Nucleotide Archive under the accession numbers LT669770 to LT669786 and LT671678 to LT671744.

Alignments of SP proteins were performed with MAFFT (Katoh et al., 2002) or Clustal Omega (Sievers et al., 2011). Using the package Seaview v4.4.1 (Gouy et al., 2010), and following alignment editing with Gblocks if appropriate, both Neighbor-Joining and Maximum-Likelihood trees were constructed. For the latter, the LG amino-acid substitution model was corrected by a Γ-law distribution with four categories of evolution rates; both invariable sites and the α-parameter of the Γ-law distribution were optimized according to the data. Branch support was estimated with aLRT (Anisimova and Gascuel, 2006).

2.3. Reconstruction of the evolutionary scenario

To reconstruct the evolutionary scenario of the SP protein family in the Yarrowia clade, a species tree based on the concatenation of 912 proteins (Meunchan et al., 2015) and a Sugar Porter tree built from an alignment of 110 proteins were reconciled with synteny data. A parsimonious approach was used to minimize the number of duplications and losses of SP genes.

2.4. Cloning of candidate transporter genes

Using genomic DNA and the primers listed in Table S1, we amplified 24 different SP genes and 8 polymorphic genes that originated from either strain W29 or strain H222, and 7 SP genes from *C. hispaniensis*. As indicated in Table S2, PCR fragments were cloned into one of three replicative plasmids in *S. cerevisiae*. The pRS426 plasmid containing the $TEF1_{Sc}$ promoter was used for our exhaustive screening procedure and was constructed from pRS426-ADH1 (Mumberg et al., 1995) as described in a footnote of Table S2. Recombinant plasmids were introduced into the *S. cerevisiae hxt*-null mutant EBY.VW4000 using the LiAc transformation protocol and selected on minimal YNBs media with 2% maltose that was supplemented for auxotrophies. The quality of all constructs and the presence of the target genes in the transformants were verified by sequencing and PCR, respectively.

2.5. Growth tests for recombinant S. cerevisiae

The ability of the recombinant EBY.VW4000 strains to grow on a given hexose following transporter expression was evaluated by drop-test assays or by monitoring growth in flask cultures via OD at 600 nm. To increase and standardize plasmid copy number, these strains were grown for three successive 24 h precultures in 5 mL of YNBs medium with 2% maltose. In each case, exponentially growing cells from the final preculture were centrifuged and washed twice with sterile distilled water before being used as inocula. A 5-µl aliquot of cell suspension was standardized to an OD_{600} of 1 and 10-fold serial dilutions (from 10^0 to 10^{-5}) were spotted onto the indicated agar media and incubated for 3 to 7 days at 28 °C. As expected, the transformed strains grew on maltose, which is taken up by a specific transporter which is present in the EBY.VW4000 strain (Wieczorke et al., 1999). A growth index, representing normalized growth fitness, was calculated as the most dilute spot (with 10^0 represented as 1 and 10^{-5} as 6) for which at least two colonies were observed on the tested sugar (reduced by 0.5 for tiny colonies), divided by the most dilute spot (same notation as above) for which at least two colonies were observed on the control plate with maltose. To avoid numbers below 1, growth index values were arbitrarily multiplied by 6, the number of spotted drops. Standard deviations did not exceed 10% of the average except in a few conditions of medium- to low-growth of recombinant *S. cerevisiae*: 11 to 20% for the mutants that expressed *YHT5* on 2% fructose, *YHT6* on 0.1% or 1% mannose, *YHT2* on 0.5% to 2% fructose, and D0111 on 0.5% to 2% glucose; 21 to 40% for mutants that expressed *YHT5* on 2% mannose and every concentration of galactose, *YHT2* on 1% and 2% mannose.

2.6. Y. Lipolytica strains disrupted for transporter genes

Hexose transport genes were deleted in PO1d derivatives following the scheme of selection and marker rescue illustrated in Fig. S1. The general principle for gene deletion was the replacement of the target locus, via double cross-over homologous recombination, with a NotI-digested cassette that consisted of a selection marker flanked by 0.8 to 1 kb of DNA upstream (P) and downstream (T) of the gene to be deleted (Fickers et al., 2003). To permit successive chromosome modifications, marker rescue was performed using the lox sites bordering the markers and the Cre recombinase expressed from the replicative plasmid pJME547, as described (Fickers et al., 2003). PCR reactions were carried out with Pyrobest DNA polymerase (Takara); the oligonucleotides for generating P and T fragments are listed in Table S1. Briefly, after column purification (QIAquick, Qiagen), a second PCR was performed in order to fuse the two PCR fragments, then the resulting product was cloned into PCR-BluntII-TOPO (Invitrogen) and the appropriate marker was inserted, using I-SceI, to produce the disruption cassettes. Targeted gene disruption after LiAc transformation was checked by PCR reactions which probed both for cassette integration at the correct locus and the absence of the targeted gene. The *Y. lipolytica* recipient strains – JMY2033 (Lazar et al., 2013) and its derivative JMY3536, in which auxotrophic markers had been rescued, and Δ*ku70* JMY2394 (Verbeke et al., 2013) – and plasmids that provided the required auxotrophic markers – pJME803 (=JMP62-lox-URA3) (Nicaud et al., 2002), pJME1226 (Lazar et al., 2013), and pINA62 (Gaillardin and Ribet, 1987), kindly provided by B. Treton – were from our collection and have been previously described (Fig. S1).

2.7. Growth tests for Y. lipolytica

Growth test experiments were carried out as drop-test assays or in liquid culture in microplates, using prototrophic strains. Cells were pregrown overnight in YPG, washed, and suspended in YNB medium without a carbon source. They were spotted on agar plates as described above for drop-test assays with *S. cerevisiae* and plates were incubated at 28 °C for at least 2 days. Microplates (96-well Greiner Bio-One polystyrene culture plates, with a sterile flat bottom and a lid) were incubated in a Synergy MX microplate reader (BioTek Instruments, Colmar) at 28 °C under fast and continuous shaking, for monitoring absorbance at 600 nm. Wells were filled with 200 µl YNB minimal medium inoculated to an initial OD_{600} of 0.2.

2.8. Detection of transcripts

The transcription of genes involved in glucose and fructose transport or phosphorylation was detected by end-point RT-PCR following the extraction of RNA from cells grown in flasks (for general cultivation see 2.1) or bioreactors. Bioreactor batch cultures were carried out for 148 h in 5-L BIO-STAT B-PLUS reactors (Sartorius, Frankfurt, Germany) filled with 2 L of medium which contained 100 g sucrose, 1.7 g YNB, 1.5 g NH_4Cl, 0.7 g KH_2PO_4, and 1.0 g $MgSO_4{\cdot}7H_2O$ per 1 L of tap water. The aeration rate was 1.0 vvm, stirring was at 800 rpm, and pH was automatically regulated at 6.8 with NaOH. Inoculation was performed as described in (Lazar et al., 2014), from cells which were pregrown for 48 h in YNB glucose-based medium and suspended in the inoculation medium composed of 50 g sucrose, 1.5 g NH_4Cl, 1.0 g YE, and 1.0 g peptone per 1 L of tap water. At least two independent cultivations were performed; bioreactors were sampled once for time-course transcription analysis.

RNA was extracted from frozen collected samples using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized as described in (Lazar et al., 2014). Semi-quantitative PCR reactions were performed using the GoTaq DNA Polymerase Kit (Promega) and the specific primers listed in Table S1. The actin gene was used as an internal standard. One gel-view sliced at the expected size for each PCR product is presented. For each gene, a band of the expected size, ranging from 160 to 210 bp, was amplified from genomic DNA (not shown). In RT-PCR experiments, some fuzzy bands corresponding to small products occasionally appeared; these are not shown in the presented pictures.

3. Results

The predicted proteome of the *Y. lipolytica* E150 reference strain (as well as draft genomes of W29 and H222) was mined for high-capacity fructose transporters of the DHA1 family. However, using reciprocal BLAST, we did not find any gene products that were orthologous to Ffz1 of *Z. rouxii*. This lack of orthology was supported by the fact that the gene products of the two top hits (YALI0F07062 and YALI0A15576; 32% and 30% amino-acid identity over 70% of Ffz1 protein with E-values of $5.e^{-69}$ and $9.e^{-63}$, respectively) did not exhibit hexose transport activity in *S. cerevisiae* functional complementation assays (not shown). We therefore focused our search for hexose transporters on the SP family.

3.1. The Sugar Porter family in Y. lipolytica strains E150, W29, and H222

We assessed SP genes in three different strains of *Y. lipolytica*. For E150, reexamination of the YETI database (De Hertogh et al., 2006) after successive genome curation (Génolevures consortium) and reannotation (available on the GRYC website) eventually identified 23 intact SP genes and 3 pseudogenes (Table 1), in place of the previous 27 SP genes (of which 4 were pseudogenes). Specifically, an additional truncated pseudogene, YALI0A11550, was discovered whereas two pseudogenes, YALI0AC04708 and YALI0C10571, were no more predicted. To investigate the molecular basis of fructose uptake in *Y. lipolytica*, we mined the draft genome of strains W29 (which grows slowly in fructose) and H222 (which grows as efficiently in fructose as in glucose) (Lazar et al., 2014), using as queries the E150 SP genes and pseudogenes. This identified the same set of (pseudo)genes in W29, whereas the YALI0C04686 pseudogene in E150 was found to be an intact gene in H222 (Table 1). Of the 23 genes shared among all three strains, 8 exhibited some polymorphism at the protein level between strains W29 and H222, with at least one amino-acid (aa) variation (Table 1).

Overall, SP protein sequences were quite diverse, with 11% to 84% pairwise aa identity (24% to 93% similarity) between different SPs within a given strain of *Y. lipolytica*. This is similar to what was observed in both *S. cerevisiae* and *K. lactis* (8–11% for the lowest pairwise values, up to 99–100% identity within both species). As exemplified in *S. cerevisiae*, the SP family comprises proteins seemingly dedicated to hexose transport (Hxt1-7, Gal2), but also transporters for di- or tri-saccharides (e.g., maltose transported by Mal11, Mal31, Mph2, Mph3), or for aliphatic or cyclic polyols (e.g., glycerol, STL1; inositol, IRT1 and IRT2), as well as transporters of unknown function. To help identify candidates for hexose transporters by homology with the two well-studied species *S. cerevisiae* and *K. lactis*, we conducted a phylogenetic analysis of SP proteins from *Y. lipolytica* and these two species. We found that proteins from *Y. lipolytica* were distributed among multiple clusters (clusters A to F, Fig. 1), several of which harbored proteins able, to some degree, to transport a hexose sugar. Main hexose transporters of *S. cerevisiae* and *K. lactis* were found in cluster A, whereas high-affinity glucose transporters from *K. lactis* were also found in cluster F. Cluster C harbors, among proteins of other function, the fructose symporters Fsy1 and Frt1 in *S. cerevisiae* strain EC1118 (Galeote et al., 2010) and *K. lactis*, respectively. In addition, three of the maltose permeases in *S. cerevisiae*, found in cluster D, reportedly transport glucose in addition to their preferred substrate (Wieczorke et al., 1999). Because the phylogenetic placement of the *Y. lipolytica* SP proteins was not informative enough, at this stage, to confidently narrow down our list of candidate hexose transporters, we decided to exhaustively investigate the hexose transport abilities of all 24 intact SP genes.

Table 1
Genes, pseudogenes and gene products of the SP family in different strains of *Y. lipolytica*.

Locus tag (strain E150)	Length (in aa)[a]	Gene name[b]	Polymorphism in W29 (aa E150/position/aa W29)	Polymorphism in H222 (aa E150/position/aa H222)
YALI0A01958	533		H50R, A61T, K212N	H50R, A61T, K212N
YALI0A08998	575		–	–
YALI0A11550	*pseudogene*		*pseudogene*	*pseudogene*
YALI0A14212	505		–	A23V
YALI0B00396	579		D435G, N576D	N576D
YALI0B01342	554	*YHT5*	–	L325I
YALI0B06391	545	*YHT6*	S543P	S543P
YALI0B17138	582		–	–
YALI0B21230	476		–	–
YALI0C04686	*pseudogene*		*pseudogene*	603 aa
YALI0C04730	581		–	L540H
YALI0C06424	515	*YHT1*	–	V319A
YALI0C08943	494	*YHT2*	–	T52I, P171S
YALI0C10560	*pseudogene*		*pseudogene*	*pseudogene*
YALI0C16522	578		–	–
YALI0D00132	659		–	–
YALI0D00363	539		–	–
YALI0D01111	592		–	–
YALI0D18876	536		–	–
YALI0E20427	566		–	–
YALI0E23287	533[c]	*YHT4*	–	–
YALI0F06776	532[d]		–	S429C
YALI0F18084	659		–	–
YALI0F19184	533	*YHT3*	G354C	–
YALI0F23903	540		–	–
YALI0F25553	599		–	–

[a] As referenced in GRYC database (http://gryc.inra.fr/index.php?page=home).
[b] Abbreviations of gene names proposed in this work, YHT for Yarrowia Hexose Transporter.
[c] The gene model had previously been mispredicted with a non validated intron in http://www.genolevures.org/elt/YALI/YALI0E23287g but corrected in htt://gryc.inra.fr.
[d] An isoform gene product due to alternative transcription start and splicing, as revealed by RNAseq, is also depicted and generates a 26 aa N-terminal extension. This was not the version used in this work.

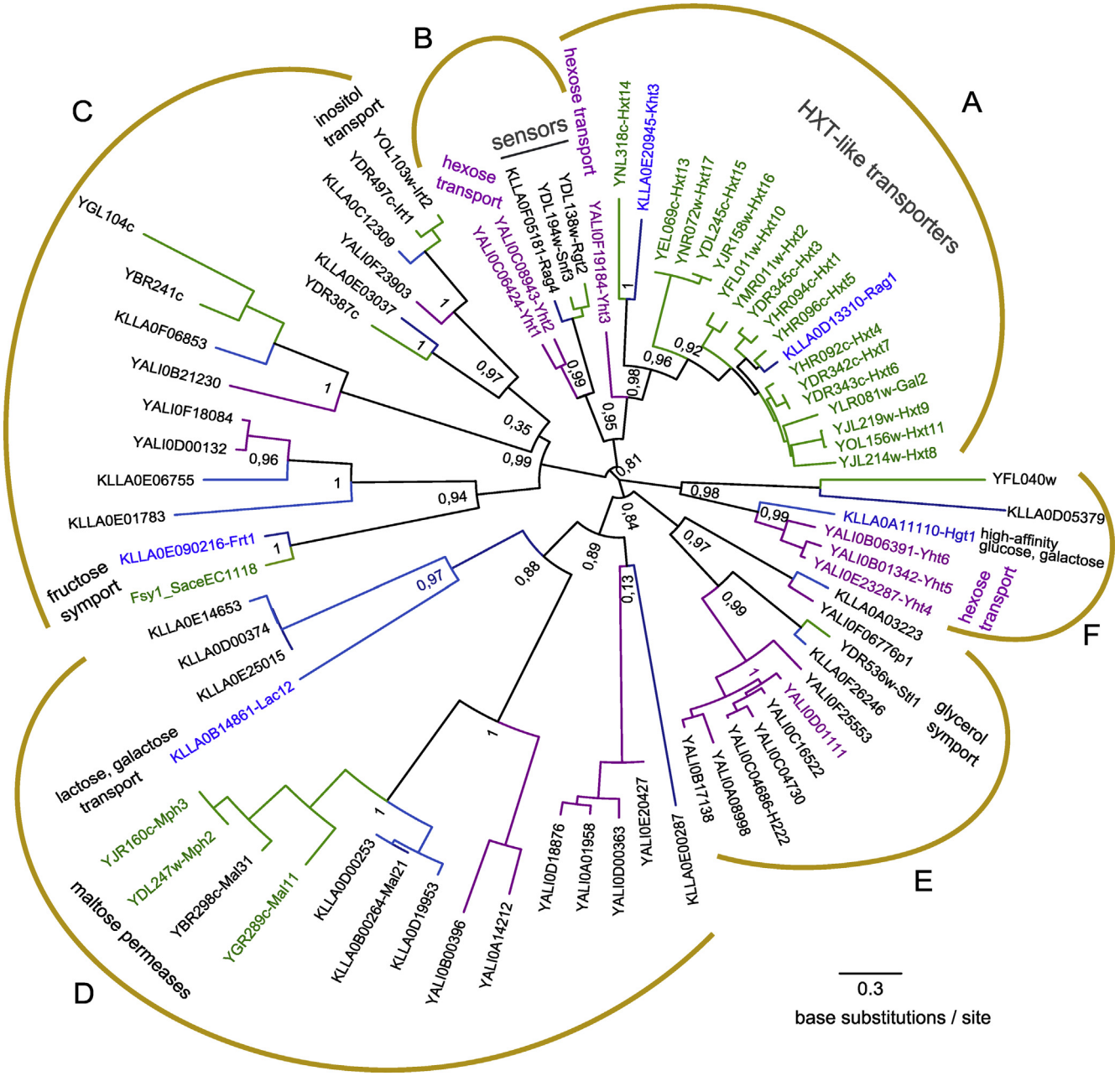

Fig. 1. Phylogenetic analysis of the SP protein family in selected yeast species. SP proteins from *S. cerevisiae* 288c, *K. lactis* NRRL Y-1140, and *Y. lipolytica* E150 were retrieved from Génolevures (GLC0002 family). The divergent YDL199c gene and the *HXT12* pseudogene were eliminated from the list, but we included both the Fsy1 fructose/H+ symporter in strain EC1118 as well as the YALI0C04686 protein from *Y. lipolytica* H222. aLRT branch support values are indicated at the nodes of the maximum-likelihood tree (omitted in external nodes), which was viewed using figtree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). Branches in green, blue, and magenta highlight *S. cerevisiae*, *K. lactis*, and *Y. lipolytica* species, respectively. Leaf labels in green and blue represent proteins from *S. cerevisiae* and *K. lactis*, respectively, that have been reported to have at least some ability for hexose transport, even if it is not the main function of those proteins. Newly experimentally determined proteins of *Y. lipolytica* are indicated in magenta.

3.2. Complementation of a S. cerevisiae hxt-null mutant reveals proteins with hexose transport activity

To characterize the hexose transport function of the SP genes of *Y. lipolytica*, we examined their capacity to rescue growth of the *S. cerevisiae* strain EBY.VW4000 on different hexoses (glucose, fructose, mannose, or galactose) at four concentrations (0.1%, 0.5%, 1%, and 2%). Without complementation by a heterologous transporter, this *hxt*-null mutant is unable to grow on hexoses due to multiple transport gene disruptions (Wieczorke et al., 1999).

As a preliminary step to optimize the detection of transporter activity, we first examined the effect of selected expression vectors on sensitivity of drop-test assays. To do this, we used the three candidate genes that encode the closest homologs to *S. cerevisiae* Hxt proteins (i.e. YALIC06424$_{W29}$, YALIC08943$_{W29}$, and YALIF19184$_{H222}$), that we each cloned into three expression vectors, which differed in the replicative origin and/or the promoter they contained (Table S2). By comparing growth of the resulting recombinant *S. cerevisiae* strains, we concluded that centromeric plasmid pRS416 (Mumberg et al., 1995) was not suited for our assays, since no growth was observed on any of the four hexoses (glucose, fructose, mannose, and galactose; data not shown). Furthermore, when carried on a 2μ plasmid (pRS426 (Mumberg et al., 1995)) the strong and constitutive p*TEF*$_{Sc}$ promoter

outperformed p*ADH1*$_{Sc}$, with which only limited complementation was observed (Fig. S2). We therefore used pRS426-TEF as the expression vector for the hexose-transport screening of all potential SP proteins identified in *Y. lipolytica* strains W29 and H222.

For six genes, which we designated *YHT1* to *YHT6* (Table 1), complementation was observed at varying degrees of efficiency depending on the identity and concentration of the sugar substrate (Fig. S3). *YHT1*, *YHT3* (the H222 allele), and *YHT4* appear to encode efficient broad-range hexose transporters, as judged by their corresponding strains' high growth index on the four hexoses in the drop-test assays (Fig. 2). Instead, *YHT2* and *YHT6* code for transporters that are seemingly dedicated to one or two hexoses (Fig. 2). *YHT5* and *YHT6* did not enable fructose uptake at any concentration in these assays. These results were further confirmed by measuring the growth rates of the *S. cerevisiae* EBY.VW400 *YHT* transformants in liquid YNB minimal media that contained glucose, fructose, or mannose at 1%. For single-*YHT* strains growing on a given sugar, the calculated growth rates were in the range of 0.046 ± 0.004 to 0.141 ± 0.016 h^{-1} (Fig. S4) and correlated well with the pattern of substrate specificity demonstrated via the drop tests. These results confirmed that these six *YHT* genes encode hexose transporters.

Among the other *Y. lipolytica* SP genes tested, only one, YALI0D01111 (referred to hereafter as D01111), was found to reproducibly confer a weak growth phenotype in drop-test assays, on glucose media only (growth index around 2 on 1% and 2% glucose). None of its closest *Y. lipolytica* homologs (Fig. 1), with which it shared 63 to 87% aa identity, could be identified as a hexose transporter in these assays. The D01111 protein appears to be related to transporters of other substrates than hexoses (e.g., SLT1$_{Sc}$, a glycerol transporter) or to SP of unknown function (Fig. 1). We therefore suspect that glucose uptake is not its main function. Very recently, D01111 was reported to participate in the uptake of cellobiose (a disaccharide of glucose) in *Y. lipolytica* (Ryu et al., 2015).

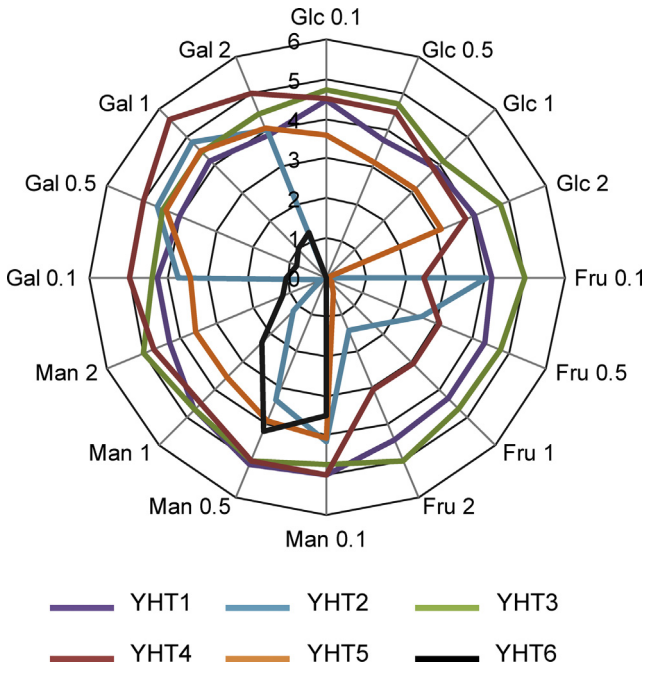

Fig. 2. Growth of *S. cerevisiae* EBY.VW4000 complemented with candidate transporter genes from *Y. lipolytica*. Results of drop-test assays summarized as growth index values. The values obtained represent growth in media containing one type of sugar at the concentration indicated (%) on the different axes. The index value indicates the last dilution at which the growth of S. cerevisiae transformants was detected (for details see Section 2.5). Reported values are the average of at least two independent experiments. Fru – fructose, Gal – galactose, Glc – glucose, Man – mannose.

3.3. Differences in hexose transport function of proteins from the two *Y. lipolytica* strains

We examined differences in transporter function between strains W29 and H222. The H222-specific SP, YALI0C04686, did not appear to function as a hexose transporter on any of the four hexoses tested (not shown). Additionally, no growth differences were detected for the eight SPs that were polymorphic between strains W29 and H222 (Table 1), with one exception: *YHT3*. For this gene, complementation of *S. cerevisiae* EBY.VW4000 with the W29 version resulted in a low-growth phenotype at low concentrations of the four hexoses tested and a moderate-growth phenotype at high concentrations of glucose and fructose. Instead, the strain transformed with *YHT3*$_{H222}$ generally demonstrated more rapid growth (Fig. S5A). This change in substrate affinity or, most probably, partial loss of activity could possibly be linked to the unique aa difference observed at residue 354, which is a cystein (C) in W29 and a glycine (G) in H222. This residue is located in the loop between transmembrane domains (TMs) 8 and 9, in the motif "GRR" which is perfectly conserved in the hexose transporters of *S. cerevisiae, K. lactis*, and strain H222 of *Y. lipolytica* (Fig. S5B). These amino acids are part of Motif A, which may occur in four different loops in transporters of the Major Facilitator Superfamily (including SPs) and was reported to be involved in conformational changes responsible for transport activity (Zhang et al., 2015).

3.4. Deletion analysis of YHT genes in *Y. lipolytica* W29

To identify the main transporters involved in the growth of *Y. lipolytica* on fructose, we constructed derivatives of strain W29 in which we deleted, individually and in combination, the *YHT* genes implicated in fructose transport (*YHT1* to *YHT4*). Growth tests were performed in a microplate reader (not shown) or as drop-test assays on plates of minimal medium that contained a given sugar as carbon source (fructose, glucose, and mannose; galactose was not tested due to the inability of *Y. lipolytica* strains to naturally grow on this sugar). The single-deletion *Δyht1* mutant demonstrated a significantly altered phenotype on fructose only. At low fructose concentrations (⩽ 0.1%), deletion of only *yht1* was sufficient to prevent the growth of mutant *Y. lipolytica* cells (Fig. 3, panel 1 at 0.1%, at 0.05% is not shown). Instead, at high fructose concentrations (⩾1%), the *Δyht1* mutant exhibited more robust growth than the WT strain (Fig. 3, panel 2 at 1.0% and 2.0%). Complementation of the *yht1* deletion with a *YHT1* gene expressed under the p*TEF*$_{Yl}$ promoter restored the WT phenotype on fructose, i.e. growth at low concentrations and less robust growth at higher concentrations (not shown). No other single-gene deletion (*yht2*, *yht3*, or *yht4*) visibly affected growth in any of the three hexoses (Fig. 3). The double Δ*yht1-2* and triple Δ*yht1-3* mutants exhibited the same phenotype as the single Δ*yht1* mutant and were not affected in their growth on glucose or mannose. In contrast, the quadruple mutant displayed a severe growth defect in all tested conditions (Fig. 3). The double deletion of *YHT1* and *YHT4* gave rise to the same phenotype as well, indicating that these two genes are the main hexose transporters in *Y. lipolytica* under laboratory conditions.

It should be noted that, in both the quadruple and *Δyht1Δyht4* mutants, residual growth could be observed at high glucose concentration depending on the culture conditions. Low growth was observed in drop-test assays (Fig. 3) and very delayed (48 h) growth was occasionally observed in microplates (not shown).

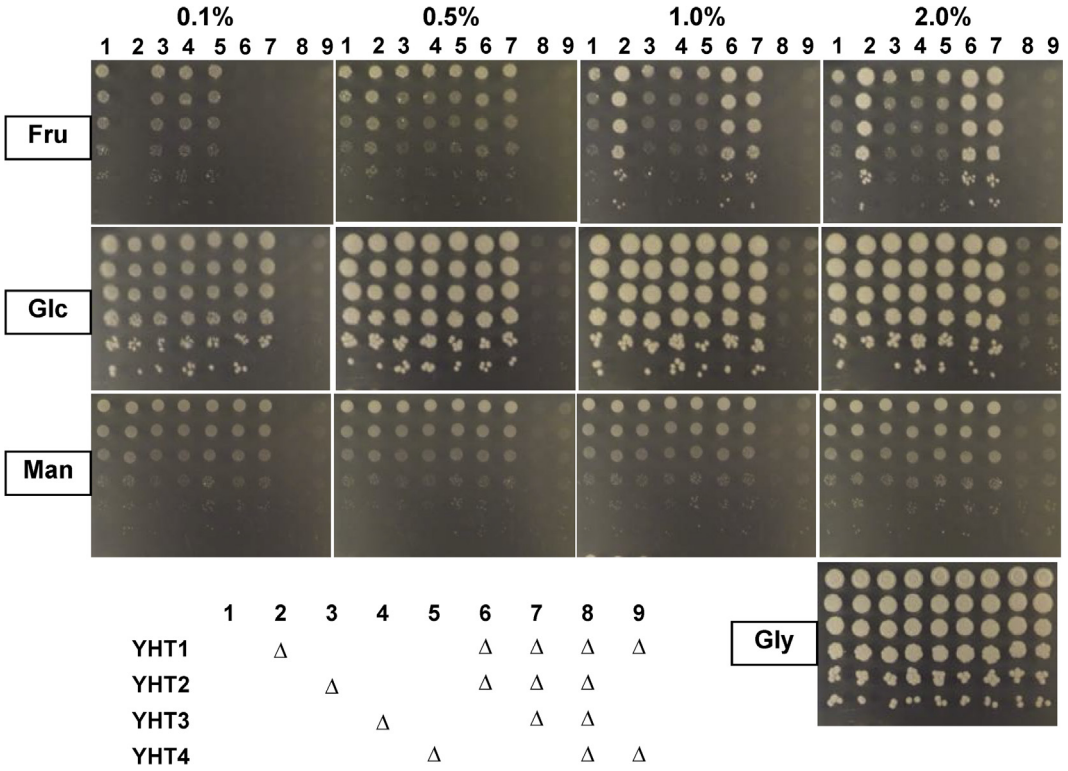

Fig. 3. Growth of *Y. lipolytica YHT* mutants on different hexoses. Drop-test assays were performed on YNB plates with glucose (Glc), fructose (Fru), or mannose (Man) at the concentration indicated above the pictures. Cell suspensions were serially diluted, and 5 µl of each dilution was spotted from top to bottom. Glycerol (Gly) was used as a positive control for growth. Strains are WT W29 (1); and, as depicted in the summary table, the mono-disrupted strains Δ*yht1* (2), Δ*yht2* (3), Δ*yht3* (4), and Δ*yht4* (5); the double mutant Δ*yht1*-2 (6); the triple mutant *Δyht1-3* (7); the quadruple mutant Δ*yht1-4* (8), and the double mutant Δ*yht1*Δ*yht4* (9). Results from one of two independent experiments are shown.

3.5. Transcription of transporter and hexokinase genes in W29 and H222 during growth in fructose with varying concentrations of glucose

Transcriptional studies are useful in elucidating which of several players are active under specific conditions. A parallel study on the metabolism of galactose in the *Y. lipolytica* W29 background revealed that *YHT1*, *YHT4*, and, to a much lesser degree, *YHT5* were transcribed in glucose and in galactose media (Lazar et al., 2015). To evaluate the function of these genes in other hexose-containing media and to examine fructose metabolism in *Y. lipolytica* in greater depth, we explored the regulation of all seven transporter genes (six *YHT* + D01111) in the presence of glucose and fructose. Specifically, we wanted to see if these genes were responsible for inter-strain growth differences in fructose or the preferential uptake of glucose over fructose. To this end, we investigated their transcription in both strain W29 and strain H222 growing in fructose-containing media.

First, an RT-PCR analysis was carried out during growth in minimal medium that was supplemented only with fructose at 0.1 or 1%. Transcription profiles were very similar for both natural isolates (Fig. 4). *YHT1* and *YHT4* were the only two genes to be consistently transcribed in fructose. Transcripts of *YHT5* and D01111 were sporadically detected, possibly indicating their presence at a low abundance, whereas transcripts of *YHT2*, *YHT3*, and *YHT6* were not detected at all. These results are consistent with those of the gene deletion analysis performed in W29 and suggest that *YHT1* and *YHT4* code for the main transporters involved in growth on fructose in H222 as well.

Second, to investigate the possible regulation of these genes by glucose or fructose, which could explain the preferential uptake of glucose over fructose, we examined their transcription in a more-

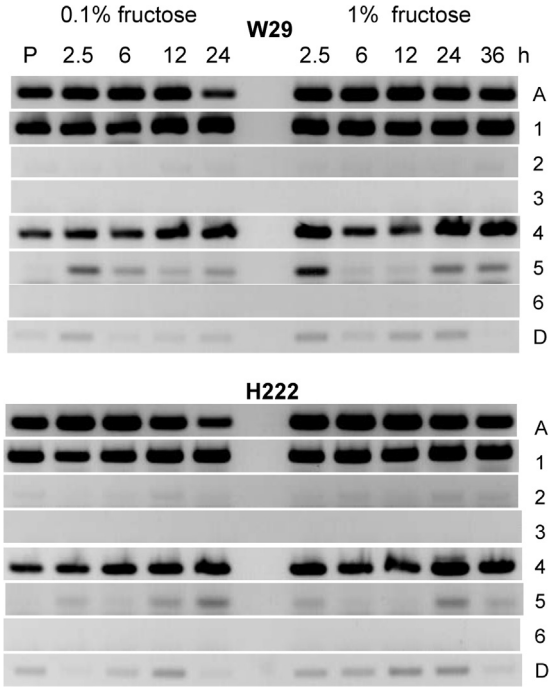

Fig. 4. Transcription profiles of *YHT* and *D01111* genes of *Y. lipolytica* strains W29 and H222 during growth in fructose. Cultivation was performed in YNB medium supplemented with either 0.1 or 1% fructose. Transcripts were detected by RT-PCR in the preculture just before inoculation (P) or after inoculation at the time indicated above the wells (h). Tested genes were as follows: A, actin; 1 to 6, YHT1 to YHT6, respectively; D, D01111. Upper panel represents profile from strain W29 and lower profile represents strain H222.

complex environment, a bioreactor in which cells were grown in sucrose medium. To do this, W29 and H222 derivative strains efficiently secreting invertase were constructed (Lazar et al., 2013). Due to continuous hydrolysis of sucrose in the medium and uptake of the resulting monosaccharides into the cell, concentrations of glucose and fructose in the medium changed over time. This provided an interesting environment in which to examine the impact of gene regulation on sugar uptake. Early on, we observed rising concentrations of glucose and fructose, indicating that sucrose hydrolysis was occurring faster than the uptake of the released sugars (Fig. 5). Glucose uptake began directly from the start of cultivation, whereas fructose was consumed only after glucose was completely (W29) or mostly (H222) depleted, in line with previous reports of *Y. lipolytica*'s preference for glucose. The transcription profiles of different genes, which were similar between the two strains (Fig. 5), could be divided into two types of patterns. The first concerned *YHT1*, *YHT4*, and D01111, whose transcripts were detected continuously during cultivation. The second one concerned *YHT2*, *YHT3*, and *YHT6*, whose transcripts were essentially detected only in the stationary phase. *YHT5* exhibited a unique pattern, as its transcripts were continuously detected but increased in abundance at the onset of the stationary phase.

Following their transport into the cell, hexose sugars such as glucose and fructose must be phosphorylated before they can be used in glycolysis and thus consumed by the cell. For this, *Y. lipolytica* reportedly harbors a glucokinase (Glk1), which is responsible for most of the glucose phosphorylation activity in the cell, and a hexokinase (Hxk1), which is responsible for all fructose phosphorylation (Petit and Gancedo, 1999). Transcripts of both genes were detected throughout the time course of our bioreactor study (Fig. 5). These results indicate that genes responsible for fructose consumption in *Y. lipolytica* are transcribed even if glucose is present.

3.6. Evolutionary dynamics of the SP family and the hexose transporter genes in Yarrowia and related species

Unlike in *S. cerevisiae*, in *Y. lipolytica*, genes for hexose transport are found in three separate phylogenetic clusters (Fig. 1), and the fact that the function of many of these proteins remains cryptic could be the result of recent adaptation and diversification in substrate range. Instead, because core hexose transporters fulfill an essential function in the cell, they may be under intense stabilizing selection and therefore, be conserved through evolution. We thus decided to investigate the conservation and evolution of these transporter genes, and the SP family as a whole, in several related yeast species. To do this, we analyzed the complete sequenced genomes (C. Neuvéglise, H. Devillers, S. Michely, unpublished results)

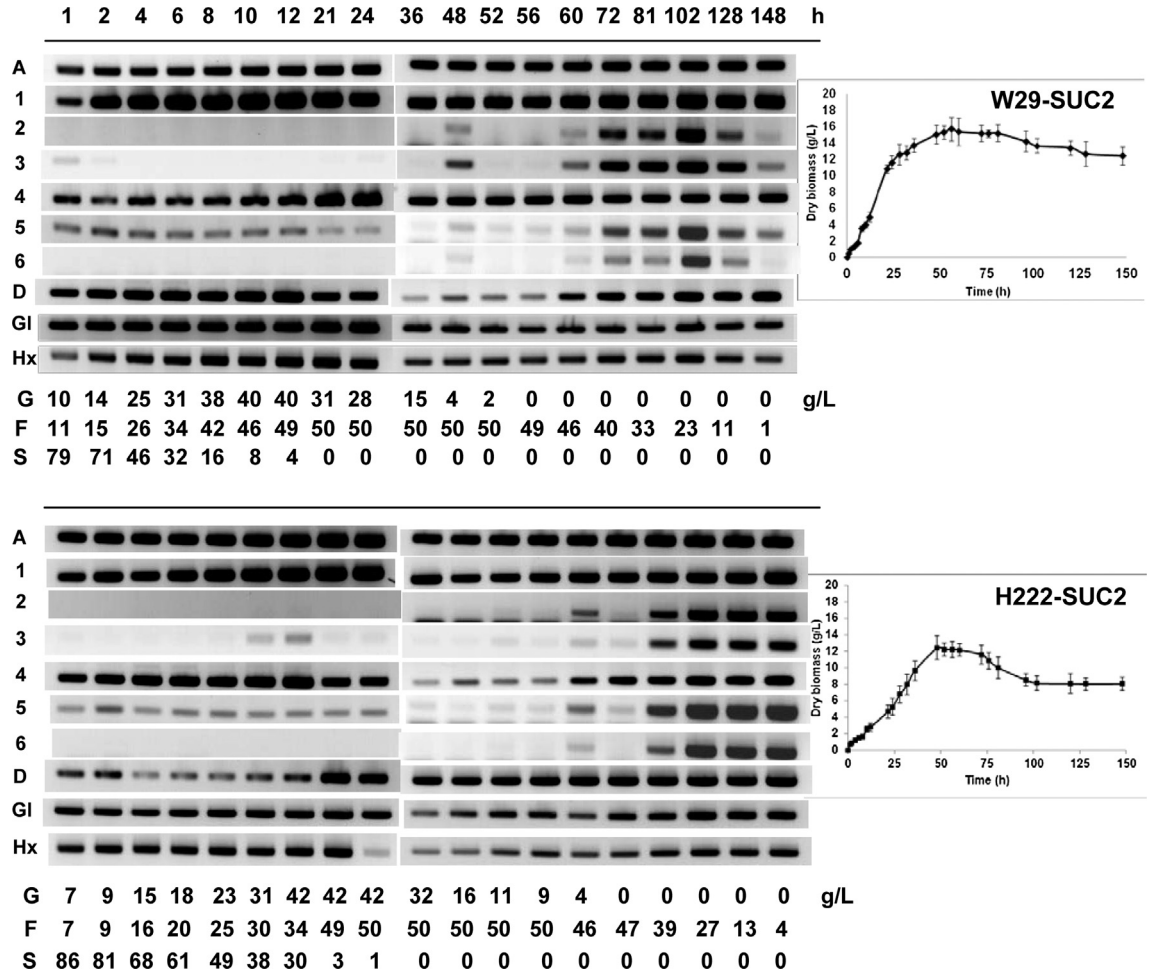

Fig. 5. Transcription profile of selected genes involved in glucose and fructose assimilation during growth of *Y. lipolytica* in sucrose. Derivatives of strains W29 (upper panel, W29-SUC2) or H222 (lower panel, H222-SUC2) that carried the invertase expression cassette $pTEF_{Yl}$-preSUC2-$SUC2_{Sc}$ (Lazar et al., 2013) were grown in bioreactors filled with YNB sucrose medium. Sampling time (in hours) is indicated at the top of the figure. Concentrations of the different sugars in the medium – sucrose (S), glucose (G), and fructose (F) – are reported underneath each panel (g/L) and were measured by HPLC with an Aminex HPX87H column coupled with an RI detector as described (Lazar et al., 2013). Transcripts of the selected genes were detected by RT-PCR: A, actin; 1 to 6, YHT1 to YHT6, respectively; D, D01111; Gl, GLK1; Hx, HXK1.

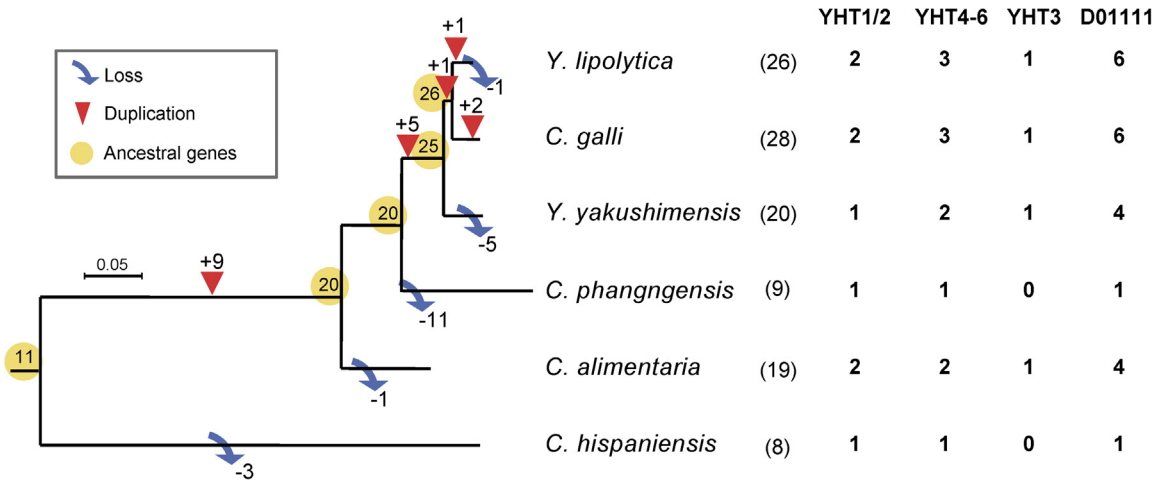

Fig. 6. Evolutionary scenario of SP genes in the *Yarrowia* clade. The number of duplication and loss events affecting these genes or pseudogenes are indicated on the branches of the species tree. Numbers of SP (pseudo)genes per genome are indicated within brackets. The counts of (pseudo)genes in the four lineages of interest are reported on the right.

of four species closely related to *Y. lipolytica* (namely, *Candida galli*, *Yarrowia yakushimensis*, *Yarrowia phangngensis*, *Candida alimentaria*) and one species at the root of this clade (*Candida hispaniensis*). All these species grow well on both glucose and fructose (Michely et al., 2013). We used BLAST to retrieve the SP genes and pseudogenes in each species, then constructed phylogenetic trees of the relationships among the recovered genes (Fig. S6A). The most-parsimonious evolutionary scenario contained 11 lineages of SP genes. It revealed that the final number of SP (pseudo)genes was shaped in each species by several duplication or loss events (Fig. 6). Unlike the other four yeasts, which contained many more (pseudo)genes (from 19 to 28 in total) than their shared ancestor (11 in our evolutionary scenario), *C. phangngensis* and *C. hispaniensis* contained fewer, with 9 and 8 (pseudo)genes remaining, respectively. The *Y. lipolytica* genes for which we detected hexose transport ability were separated into four lineages: YHT1/2, YHT3, YHT4/5/6, and D01111 (Fig. S6A). DO1111 is a member of the largest lineage (22 members), while *YHT3* belongs to the smallest one (4 members). *YHT3* aside, at least one (pseudo)gene from each hexose transporter lineage was found in each of the yeast species studied, including the basal *C. hispaniensis* (Fig. 6; Fig. S6A). More precisely, when we took into account the conservation of gene neighborhoods (synteny) between *Y. lipolytica* and *C. hispaniensis*, we were able to determine that the ancestral versions in the YHT1/2 and YHT4/5/6 lineages were *YHT1* and *YHT4*, respectively (Fig. S6B and C). However, *YHT4* was found as a pseudogene in *C. hispaniensis*, and therefore does/should not produce a functional transporter protein. Because the number of intact SP genes in *C. hispaniensis* is so small (7), we easily evaluated them for their ability to complement the hexose transport defect of the *S. cerevisiae hxt*-null mutant. Of the *C. hispaniensis* SP genes, only *YHT1* (OLHI0E5622) was able to complement the growth of *hxt*-null *S. cerevisiae* on hexose, and it demonstrated a similar substrate-range profile as *YHT1* of *Y. lipolytica* (data not shown). This was consistent with our findings that Yht1 is one of the major hexose transporters. In addition, although the function of *YHT4* was apparently lost in *C. hispaniensis*, the orthologous gene (confirmed by synteny) is still present in *C. phangngensis*; together with *YHT1*, they are the only two genes of the YHT lineages in this species (Fig. S6B and C).

4. Discussion

This work represents the first time that experimental evidence has been used for the large-scale identification of hexose transporters in the oleaginous yeast *Y. lipolytica*. We favored a two-step approach, first revealing the transporters' function in a heterologous host and then examining their role in the growth of *Y. lipolytica* in monosaccharide-based media. A previous study by Young et al. (2011) that examined selected candidate transporters using a similar strategy of complementation assays of the *hxt*-null mutant *S. cerevisiae* EBY.VW4000 did not detect the ability of either YALIC06424 or YALIC08943 to take up fructose, which instead was revealed by our analysis. As reported here, the low-copy-number centromeric plasmid pRS416 (Mumberg et al., 1995) or the use of the sugar-dependent p$ADH1_{Sc}$ promoter to drive cloned genes may limit their expression and therefore growth complementation. This study also differs in providing the results of a systematic analysis which uses four different hexoses at four concentrations in order to accurately indicate the substrate range of the candidate hexose transporters.

4.1. Physiology of hexose uptake in Y. lipolytica

Six genes in the SP family were considered to encode *bona fide* hexose transporters in our complementation assays and were named *YHT*, for Yarrowia Hexose Transporter (*YHT1* to *YHT6*, Table 1). Four of these proteins promoted growth of the complemented *S. cerevisiae* in at least three out of the four hexose sugars tested regardless of their concentration in the medium (excepting the Yht3 variant in W29, Fig. S5). Yht2 and Yht6 exhibited narrower substrate specificity, and growth rescue depended, at least partly, on the sugar concentration in the medium. Further work is needed to determine the K_m and V_{max} parameters of the hexose transporters of *Y. lipolytica* and fully understand the basis of differences in growth of the complemented strains. Such apparent weaker growth in medium-to-high sugar concentrations has already been observed for high-affinity symporters such as Fsy1 of *Z. rouxii*, which reportedly complemented growth of the *hxt-null* EBY.VW4000 strain better on 0.25% than on 10% fructose medium (Leandro et al., 2013) or for Fsy1 from *S. cerevisiae* EC1118 in our tests (Fig. S3). Generally speaking, the SP protein family harbors proteins with different mechanism of transport (passive diffusion, symport or antiport). To the best of our knowledge, this mechanism cannot be deduced from specific motifs in protein sequence. It should be noted however that no protein closely related to the Fsy1 fructose/H+ symporter could be identified in any of the three sequenced *Y. lipolytica* strains used in our study. Although Fsy1 has been subjected to extensive intra-kingdom

horizontal gene transfer, its absence here is consistent both with its patchy distribution in yeasts and its absence in the *Y. lipolytica* E150 strain, as observed by Coelho et al. (2013).

Disruption mutants revealed that the growth of *Y. lipolytica* in glucose, mannose, and fructose relied on both Yht1 and Yht4 transporters. Their important functions depend on their expression levels, as judged by RT-PCR, and, to a lesser degree, in the efficiency of sugar transport conferred by the proteins. *YHT5* probably plays a secondary role in this regard, as its transcripts were detected, at least weakly, while the strain was growing in fructose, sucrose (this study), or glucose (Lazar et al., 2015), but it is probably less efficient –or not efficient at all towards fructose, as judged by the weaker growth complementation of its *S. cerevisiae* transport mutant.

Turning specifically to fructose assimilation, it is interesting that the same set of fructose transporters appears to be used (expressed at the transcriptional level) in both strains W29 and H222, as these strains differ in their growth rates in fructose. This similarity in transporter gene expression indirectly confirms the importance of sugar phosphorylation by hexokinase for efficient fructose metabolism, since overexpression of the hexokinase gene reportedly enabled W29 to grow as fast as H222 in both fructose and glucose (Lazar et al., 2014). Either one of the Yht1 or Yht4 transporters was sufficient to support the growth of *Y. lipolytica* W29 on media containing any of the three hexoses, except on fructose at concentrations below 0.1%. In this situation, Yht1 was necessary and sufficient. This may be linked to the lower affinity for fructose of the second transporter Yht4, if the growth complementation index obtained in *S. cerevisiae* faithfully reflects relative transporter kinetics (as has been assumed for growth rates in other studies using similar heterologous hosts that expressed transporters (Young et al., 2011, 2014)). Conversely and unexpectedly, the presence of *YHT1* had a negative effect on the growth of W29 in 1% fructose. This effect might be related to the particular context of the W29 strain, in which a low level of hexokinase activity prevents good growth at high fructose concentrations (Lazar et al., 2014), and possibly leads to an unfavorable overflow of fructose in the presence of an efficient transporter. *Y. lipolytica* strains expressing a single *YHT* gene under the p*TEF* promoter as sole hexose transporter, along with the p*TEF-HXK1* cassette for efficient fructose metabolism, came in support to these hypotheses (Lazar and Crutz-Le Coq, unpublished results): in fructose 0.1%, the single-*YHT4* strain exhibited very slow growth (but normal growth in glucose), while the isogenic *YHT1* strain showed the same growth rate as observed for the control strain (overexpressing hexokinase and native set of transporters); in fructose 1%, the growth of single-*YHT1* strain was also similar to the control. Regarding *Y. lipolytica* preferential uptake of glucose over fructose when growing in a mixture of both sugars, which is a physiologic hallmark in both H222 and W29 strains (Lazar et al., 2013; Moeller et al., 2012), our study shows that this preference does not rely on a transcriptional switch-off of the genes involved in fructose consumption, i.e. the fructose transporters Yht1 and Yht4 and hexokinase.

4.2. Evolution and distribution of yeasts' hexose transporters

This study provides new insights into the evolution and distribution of hexose transporters in non-conventional yeasts. With the exception of the Ffz fructose transporters, which are possibly restricted to fructophilic yeasts closely related to the genus *Zygosaccharomyces* (Cabral et al., 2015), the SP family comprises most, if not all, of the hexose transporters characterized so far in yeasts. Within this family, hexose transporter proteins have a polyphyletic origin (Fig. 1). As exemplified by the case of *Y. lipolytica* in this work, the major yeast hexose transporters can be divided into at least three distinct phylogenetic clusters. One of these clusters (cluster A in Fig. 1), includes the well-known Hxt-type hexose transporters of *S. cerevisiae*. Homologs of these transporters have been found in many investigated species of Saccharomycotina as well as in *Schizosaccharomyces pombe* (Lin and Li, 2011; Palma et al., 2009). Interestingly, while certain members of this group serve as transporters for growth on hexoses (e.g., Hxt1-7 in *S. cerevisiae*), others appear to be involved in the transport of other sugar-like substrates or even in various cellular functions. Among the eight *S. pombe* proteins homologous to the Hxt proteins (Lin and Li, 2011), Ght4 was shown to transport gluconate instead of glucose (Heiland et al., 2000). Moreover, the main molecular function of some of the *S. cerevisiae* Hxt proteins (Hxt13, Hxt15, Hxt16, Hxt17), which had remained unclear for more than a decade, has just been determined to be that of polyol transporters (Jordan et al., 2016). More intriguing still is the fact that Hxt13 was assigned a role as an efflux pump that is able to mediate resistance to an antifungal agent (Biswas et al., 2013). Thus, the expansion of this group of *HXT*-like genes in *S. cerevisiae* and *S. pombe* probably reflects, at least in part, a diversification of substrate range, in addition to possible fine tuning of hexose uptake in various environments. Interestingly, the *S. cerevisiae HXT8* to *HXT17* genes (except HXT10) are located around subtelomeric regions, unlike the major hexose transporter genes (Lin and Li, 2011).

The second cluster (cluster B) is well known for containing hexose sensors from different species (Palma et al., 2009). In *Y. lipolytica*, Yht1 and Yht2 are closely related to but distinct from the branch of sensors known in *K. lactis* and *S. cerevisiae* (Fig. 1). We provide experimental evidence that both of these are true transporters in *Y. lipolytic*a, as is also the Yht1 homolog in *C. hispaniensis*, which is the sole functional hexose transporter identified in the restricted SP family of this species. This result provides additional support to the concept of a clear subdivision, at both the phylogenetic and functional level, between true sensors and their closely related transporters, which had been previously denominated as sensor-like proteins or pro-sensors (Lin and Li, 2011; Palma et al., 2009). This prosensor subgroup, which has been identified *in silico* in many different species (with the notable exception of post-WGD yeasts), also differs from true sensors in that its members lack the long C-terminal extension of sensors (Lin and Li, 2011; Palma et al., 2009). It is therefore possible that the sensors acquired this extension as they evolved their new function (Lin and Li, 2011; Palma et al., 2009). The presence of a typical Özcan motif (Ozcan et al., 1998) is, however, not required as a signature of this regulatory extension in species other than *S. cerevisiae* or its close relatives, as the Gss1 sensor of *Komagatella pastoris* does not possess this motif (Ozcan et al., 1998; Polupanov et al., 2012). In sum, it appears that *Y. lipolytica* lacks this type of glucose (hexose) sensor which is found in yeasts of the Saccharomycetaceae or CTG clade (Fig. S7).

The third cluster (cluster F in Fig. 1) is also made of two phylogenetic groups, one of which contains Yht4-6 together with Hgt1 of *K. lactis* but no member of *S. cerevisiae* (Palma et al., 2007). This group is typically referred to as HGT (Lin and Li, 2011; Palma et al., 2007) because it contains the high-affinity glucose transporter Hgt1, which has been characterized in both *K. lactis* and *Candida albicans* (Billard et al., 1996; Varma et al., 2000). However, we suggest that the larger function of this group may be in promoting basic cell growth through the transport of several different hexoses at varying concentrations, as exemplified by Yht4 in *Y. lipolytica*. Actually, although it exhibits a km of 1 mM for glucose, Hgt1 in *K. lactis* is clearly involved in the uptake of and consequent growth in glucose at a concentration of 2% and its gene is constitutively expressed independently of the glucose concentration (Billard et al., 1996). To confirm this, further investigation is needed of the role of this group in other species, such as *Scheffersomyces stipitis*, *Eremothecium gossypii*, and *Debaryomyces hansenii,* which

reportedly harbor members of this phylogenetic group (Palma et al., 2007).

4.3. Genetic redundancy in hexose transporters

Genes referred to as HXT, among which many hexose transporters have been identified, appeared to be more numerous in the aerobic fermenting yeasts (including *S. cerevisiae* and *S. pombe*) than in other yeast genomes (Lin and Li, 2011). Indeed, having undergone whole genome duplication, *S. cerevisiae* contains many more transporter genes than are strictly necessary, at least in laboratory conditions, for glucose or fructose uptake. The profusion is true, although to a lesser extent, in *S. pombe* (up to six proteins carrying hexose transport activity among eight Hxt-related proteins). Although *Y. lipolytica* is a non-fermentative respiratory yeast, we report here a similar level of genetic redundancy of hexose transporters (six *bona fide* genes in our study). In contrast to *S. cerevisiae,* this redundancy occurs through multiple phylogenetic origins and not via the expansion of a single gene family. This multiplicity of transporters has been both underestimated, when only the strict HXT lineages were taken into account (one gene) (Lin and Li, 2011), or probably overestimated, when the whole group of SPs was considered (up to 23 putative genes for glucose transport depending on claimed selectivity) (Ryu et al., 2015). It cannot be ruled out, though, that the hexose transport ability of other non-essential transporters was not detected in our or other studies due to a defect in either the expression or folding of the foreign proteins in *S. cerevisiae*. The question of physiological role and substrate range of transporters proves to be a complex one. Further work is needed to determine if any of the Yht transporters are able to transport other substrates, pentoses as an example. Indeed, hexose transporters of other species, such as Hxt7, Gal2, or Hxt5 of *S. cerevisiae* (Sedlak and Ho, 2004) are also known to transport xylose. This may also be the case in *Y. lipolytica*, as Young et al. (2014) detected very weak growth complementation in xylose for *YHT1* (YALI0C06424) and *YHT6* (YALIB06391) in an *hxt*-null mutant of *S. cerevisiae* engineered for use of xylose. This will be part of future efforts necessary to elucidate the role of the remaining SP transporters in *Y. lipolytica* whose function remains cryptic but whose genes were reported to be transcribed after glucose depletion (this study) or in different sugar media (Ryu et al., 2015).

4.4. Conclusions

Through the characterization of a set of transporters in *Y. lipolytica,* which diverged long before the model yeast *S. cerevisiae*, we were able to shed light on a new reservoir of transporter genes and provide experimental support for a better understanding of the evolution and regulation of hexose transport function across yeast species. By pointing out the prominent role as a broad-range hexose transporter of a gene phylogenetically related to *S. cerevisiae* hexose sensors and by revealing the importance of another group of hexose transporter genes, distinct from *S. cerevisiae HXT* lineages, our results will help to identify hexose transporters in their diversity among yeasts and will aid future studies of comparative genomics.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2017.01.001.

References

Altschul, S.F. et al., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. 55, 539–552.
Barth, G., Gaillardin, C., 1996. Yarrowia lipolytica. In: Wolf, K. (Ed.), Non Conventional Yeasts in Biotechnology, a Handbook. Springer-Verlag, Berlin, Germany, pp. 313–388.
Billard, P. et al., 1996. Glucose uptake in *Kluyveromyces lactis*: role of the HGT1 gene in glucose transport. J. Bacteriol. 178, 5860–5866.
Biswas, C. et al., 2013. Functional characterization of the hexose transporter Hxt13p: an efflux pump that mediates resistance to miltefosine in yeast. Fungal. Genet. Biol. 61, 23–32.
Busti, S. et al., 2010. Glucose signaling-mediated coordination of cell growth and cell cycle in *Saccharomyces cerevisiae*. Sensors (Basel) 10, 6195–6240.
Cabral, S. et al., 2015. Occurrence of *FFZ* genes in yeasts and correlation with fructophilic behaviour. Microbiology 161, 2008–2018.
Coelho, M.A., et al., 2013. Extensive Intra-Kingdom Horizontal Gene Transfer Converging on a Fungal Fructose Transporter Gene. Plos Genetics. pp. 9.
De Hertogh, B. et al., 2006. Emergence of species-specific transporters during evolution of the hemiascomycete phylum. Genetics 172, 771–781.
Fickers, P. et al., 2003. New disruption cassettes for rapid gene disruption and marker rescue in the yeast *Yarrowia lipolytica*. J. Microbiol. Methods 55, 727–737.
Gaillardin, C., Ribet, A.M., 1987. *LEU2* directed expression of beta-galactosidase activity and phleomycin resistance in *Yarrowia lipolytica*. Curr. Genet. 11, 369–375.
Galeote, V. et al., 2010. *FSY1*, a horizontally transferred gene in the *Saccharomyces cerevisiae* EC1118 wine yeast strain, encodes a high-affinity fructose/H+ symporter. Microbiology 156, 3754–3761.
Gouy, M. et al., 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27, 221–224.
Heiland, S. et al., 2000. Multiple hexose transporters of *Schizosaccharomyces pombe*. J. Bacteriol. 182, 2153–2162.
Jordan, P. et al., 2016. Hxt13, Hxt15, Hxt16 and Hxt17 from Saccharomyces cerevisiae represent a novel type of polyol transporters. Sci. Rep. 6, 23502.
Katoh, K. et al., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucl. Acids Res. 30, 3059–3066.
Kurtzman, C.P., 2011. *Yarrowia* van der Walt & von Arx (1980). In: Kurtzman, C.P. et al. (Eds.), The Yeasts, a Taxonomic Study. Elsevier B.V, London, UK, pp. 927–929.
Lazar, Z. et al., 2014. Hexokinase-A limiting factor in lipid production from fructose in *Yarrowia lipolytica*. Metab. Eng. 26, 89–99.
Lazar, Z. et al., 2015. Awakening the endogenous Leloir pathway for efficient galactose utilization by *Yarrowia lipolytica*. Biotechnol. Biofuels. 8, 185.
Lazar, Z. et al., 2013. Optimized invertase expression and secretion cassette for improving *Yarrowia lipolytica* growth on sucrose for industrial applications. J. Ind. Microbiol. Biotechnol. 40, 1273–1283.
Leandro, M.J. et al., 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, 601–608.
Leandro, M.J. et al., 2013. ZrFsy1, a high-affinity fructose/H+ symporter from fructophilic yeast *Zygosaccharomyces rouxii*. PLoS ONE 8, e68165.
Lin, Z., Li, W.H., 2011. Expansion of hexose transporter genes was associated with the evolution of aerobic fermentation in yeasts. Mol. Biol. Evol. 28, 131–142.
Mauersberger, S. et al., 2001. Insertional mutagenesis in the n-alkane-assimilating yeast *Yarrowia lipolytica*: generation of tagged mutations in genes involved in hydrophobic substrate utilization. J. Bacteriol. 183, 5102–5109.

Meunchan, M. et al., 2015. Comprehensive analysis of a Yeast Lipase Family in the *Yarrowia* Clade. PLoS ONE 10, e0143096.
Michely, S. et al., 2013. Comparative physiology of oleaginous species from the *Yarrowia* clade. PLoS ONE 8, e63356.
Moeller, L. et al., 2012. Substrate utilization by recombinant *Yarrowia lipolytica* growing on sucrose. Appl. Microbiol. Biotechnol. 93, 1695–1702.
Mumberg, D. et al., 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119–122.
Nicaud, J.M. et al., 2002. Protein expression and secretion in the yeast *Yarrowia lipolytica*. FEMS Yeast Res. 2, 371–379.
Ozcan, S. et al., 1998. Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO J. 17, 2566–2573.
Ozcan, S., Johnston, M., 1999. Function and regulation of yeast hexose transporters. Microbiol. Mol. Biol. Rev. 63, 554–569.
Palma, M. et al., 2007. A phylogenetic analysis of the sugar porters in hemiascomycetous yeasts. J. Mol. Microbiol. Biotechnol. 12, 241–248.
Palma, M. et al., 2009. Combined phylogenetic and neighbourhood analysis of the hexose transporters and glucose sensors in yeasts. FEMS Yeast Res. 9, 526–534.
Petit, T., Gancedo, C., 1999. Molecular cloning and characterization of the gene *HXK1* encoding the hexokinase from *Yarrowia lipolytica*. Yeast 15, 1573–1584.
Pina, C. et al., 2004. Ffz1, a new transporter specific for fructose from *Zygosaccharomyces bailii*. Microbiology 150, 2429–2433.
Polupanov, A.S. et al., 2012. Gss1 protein of the methylotrophic yeast *Pichia pastoris* is involved in glucose sensing, pexophagy and catabolite repression. Int. J. Biochem. Cell Biol. 44, 1906–1918.
Ryu, S. et al., 2015. Activating and elucidating metabolism of complex sugars in *Yarrowia lipolytica*. Appl. Environ. Microbiol. 82, 1334–1345.
Sedlak, M., Ho, N.W., 2004. Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast. Yeast 21, 671–684.
Sievers, F. et al., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539.
Varma, A. et al., 2000. Molecular cloning and functional characterisation of a glucose transporter, CaHGT1, of *Candida albicans*. FEMS Microbiol. Lett. 182, 15–21.
Verbeke, J. et al., 2013. Efficient homologous recombination with short length flanking fragments in Ku70 deficient *Yarrowia lipolytica* strains. Biotechnol. Lett. 35, 571–576.
Wieczorke, R. et al., 1999. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett. 464, 123–128.
Young, E. et al., 2011. Functional survey for heterologous sugar transport proteins, using *Saccharomyces cerevisiae* as a host. Appl. Environ. Microbiol. 77, 3311–3319.
Young, E.M. et al., 2014. Rewiring yeast sugar transporter preference through modifying a conserved protein motif. Proc. Natl. Acad. Sci. USA 111, 131–136.
Zhang, X.C. et al., 2015. Energy coupling mechanisms of MFS transporters. Protein. Sci. 24, 1560–1579.