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Datamining and functional environmental genomics reassess the phylogenetics and functional diversity of fungal monosaccharide transporters

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Abstract

Sugar transporters are essential components of carbon metabolism and have been extensively studied to control sugar uptake by yeasts and filamentous fungi used in fermentation processes. Based on published information on characterized fungal sugar porters, we show that this protein family encompasses phylogenetically distinct clades. While several clades encompass transporters that seemingly specialized on specific “sugar-related” molecules (e.g. *myo*-inositol, charged sugar analogs), others include mostly either mono- or di/oligosaccharide low-specificity transporters. To address the issue of substrate specificity of sugar transporters, that protein primary sequences do not fully reveal, we screened “multi-species” soil eukaryotic cDNA libraries for mannose transporters, a sugar that had never been used to select transporters. We obtained 19 environmental transporters, mostly from *Basidiomycota* and *Ascomycota*. Among them, one belonged to the unusual “fucose H⁺ symporter” family, which is only known in *Fungi* for a rhamnose transporter in *Aspergillus niger*. Functional analysis of the 19 transporters by expression in yeast and for two of them in *Xenopus laevis* oocytes for electrophysiological measurements indicated that most of them showed a preference for D-mannose over other tested D-C6 (glucose, fructose, galactose) or D-C5 (xylose) sugars. For the several glucose and fructose-negative transporters, growth of the corresponding recombinant yeast strains was prevented on mannose in the presence of one of these sugars that may act by competition for the binding site. Our results highlight the potential of environmental genomics to figure out the functional diversity of key fungal protein families and that can be explored in a context of biotechnology.

Key Points

- Most fungal sugar transporters accept several sugars as substrates
- Transporters, belonging to 2 protein families, were isolated from soil cDNA libraries
- Environmental transporters featured novel substrate specificities

Introduction

In osmotrophic microorganisms such as *Fungi*, sugar transporters inserted in the cell membrane are at the frontline for the provision of external soluble sugars to fuel primary carbon metabolism (Gonçalves et al. 2016). These transporters are confronted to, and may cope with, rapid shifts in both external concentrations and chemical diversity of available substrates. To adjust fluxes in responses to these shifts, fungi have evolved a series of transcriptional and post-transcriptional mechanisms that modulate synthesis and turnover of such membrane proteins (Özcan and Johnston 1999; Horák 2013). Additionally, these transporters have evolved intrinsic characteristics, namely substrate ranges and affinities that specialize them on a specific set of sugars in a specific range of concentrations.

In nature, sugars are represented as soluble free mono or disaccharides that accumulate in specific plant exudates such as flower nectar, plant sap, fruit pulp, rhizodeposits or milk (Hutsch et al. 2002). However, most sugars are stored as polysaccharides constitutive of the plant biomass (e.g. cellulose, hemicelluloses, starch) (Popper and Tuohy 2010; Schädel et al. 2010). Plant biomass degradation by extracellular hydrolytic enzymes continuously generates a diversity of mostly C5 (e.g. xylose, arabinose) or C6 (e.g. glucose, galactose, mannose) monosaccharides as well as oligosaccharides (e.g. maltose, xylobiose, cellobiose, mannobiose, regarding di-saccharides) that are substrates for sugar transporters (Gunina and Kuzyakov 2015). Consequently, sugar transporter-encoding gene families are among the most diversified gene families in fungal genomes with up to 86 different members in the case of the mold *Aspergillus niger* (Peng et al. 2018).

In yeasts and filamentous fungi, playing key roles in carbon metabolism, fermentation processes, and biotechnology to achieve high conversion rates of complex substrates (e.g. plant biomass, dairy effluents), sugar transporters have been the focus of numerous studies and literature reviews (e.g. Bisson et al. 2016; Hara et al. 2017). Most noticeably, is the

characterization of the substrate specificity of most of the sugar transporters in the model and industrial yeast *Saccharomyces cerevisiae* (e.g. Bisson et al. 2016). For this species, mutant and recombinant strains unable to use either mono- or disaccharides (Wieczorke et al. 1999; Marques et al. 2017) have been selected and extensively used to characterize transporters from other fungal species by functional complementation. This has also been the case for several degradation products from cellulose, hemicellulose or pectin that *S. cerevisiae* cannot naturally metabolize, such as xylose, cellobiose, xylobiose, cellobionic acid or galacturonic acid (e.g. Galazka et al. 2010; Li et al. 2015; Sloothaak et al. 2016; Zhang et al. 2017; Protzko et al. 2018).

From a structural point of view, sugar transporters are described within the Major Facilitator Superfamily (MFS) referenced in the Transported Classification Database (TCDB, Saier et al. 2016) as TC 2.A.1. Almost all fungal sugar transporters described so far belong to the so-called Sugar Porter (SP) family (TC 2.A.1.1). More marginally, sugar acquisition through specific members of the Drug:H⁺ Antiporter-1 family (DHA1, TC 2.A.1.2) has been reported (essentially for fructose) in *Saccharomycotina* yeasts (Leandro et al. 2011; Gonçalves et al. 2016), as well as through a “Fucose: H⁺ Symporter” (FHS) family member (TC 2.A.1.7) in the case of L-rhamnose in *A. niger* (Sloothaak et al. 2016). Putative SWEET transporters (TC 2.A.123; Chen et al. 2010; Takanaga and Frommer, 2010) have also been identified in the genome of chytrid fungi (Hu et al. 2016). In what concerns the SP family, it is found in all domains of life and virtually in all eukaryotic species examined. The 3-dimensional structures of several bacterial, plant and animal members of this family have been resolved (e.g. Deng et al. 2014; Wisedchaisri et al. 2014; Paulsen et al. 2019) along with the identification of key amino acid residues interacting with the transported sugars (e.g. Young et al. 2014; Zhang et al. 2015). Furthermore, it was shown that very limited changes in the fungal SP primary sequences, obtained through directed or random mutagenesis, can alter

transport features in terms of substrate range, affinity or transport inhibition by non-transported competitive substrates (e.g. Reznicek et al. 2015; Li et al. 2016; Trichez et al. 2019). Despite this apparent wealth of knowledge regarding fungal sugar transporters, their true phylogenetic and functional diversity within the kingdom Fungi remains underexplored. Indeed, functional data available for fungal sugar transporters suffer from several potential biases. On the one hand, (i) these data have been acquired from a limited number of fungal species not necessarily representative of the phylogenetic and functional diversity of this taxonomic group. On the other hand, (ii) a large number of the characterized transporters had been selected either based on their phylogenetic proximity to already characterized transporters or based on their capacity to transport a restricted number of sugars (often glucose, xylose or fructose in the case of monosaccharide transporters).

To overcome the taxonomic bias, in the present study, we selected transporters by the functional complementation of a *S. cerevisiae* yeast mutant defective in monosaccharide transport using metatranscriptomic libraries made from polyadenylated mRNA extracted from a broadleaf (*Fagus sylvatica*) and an evergreen (*Picea abies*) forest soils. Fungal communities present in these soils encompass several hundreds of taxa distributed among several main orders belonging to *Basidiomycota*, *Ascomycota* and *Mucoromycota* (Damon et al. 2010). These taxa encompass nutritional strategies featuring either soil or litter saprotrophs, white or brown rots, plant or animal pathogens and mycorrhizal mutualists. Regarding the sugars used as "baits" to select sugar transporters, we concentrated our efforts on D-mannose. In the literature, this monosaccharide had never been specifically used to select transporters although it is widespread and abundant in natura as a component of plant biomass (mannans) or as a component of glycoproteins as for example in the fungal cell wall mannoproteins (including yeasts; see Lipke and Ovalle, 1998). Noticeably, gymnosperm cell walls are specifically enriched in mannans that represent on average 31% of the needle's hemicellulose

124 biomass (Schädel et al. 2010). It could therefore be hypothesized that fungal species that
125 specifically thrive in gymnosperm (e.g. *P. abies*) forest may express mannose-specific
126 transporters. Furthermore, in a context of biotechnology, the characterization of transporters
127 that efficiently transport mannose but no other monosaccharides could be used to engineer
128 yeast strains for an efficient use of this plant-derived sugar in a context where other sugars are
129 present.

130 Our objectives were thus (i) to update our current knowledge on fungal sugar
131 transporter functional diversification through the establishment of an exhaustive database of
132 characterized members of this fungal protein family and subsequent phylogenetic analysis of
133 the corresponding proteins, and (ii) to search for novel members possibly defining novel
134 substrate preferences (such as mannose) through an environmental genomics approach.

Materials and methods

Soil sampling, RNA extraction and cDNA library construction

Two organic matter-rich composite soil samples were collected in July 2007 in a beech (*F. sylvatica*) and in a spruce (*P. abies*) forest (Damon et al. 2010; 2011; 2012) located in central France (Breuil-Chenue site, 47°18'10''N, 4°4'44''E). More details about forests, soil characteristics and sampling strategy are given in Damon et al. (2010). Total RNA was extracted from 104 g of each frozen soil samples as previously described (Damon et al. 2010) and eukaryotic cDNAs were synthesized from 3 µg of total soil RNA using the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia). After size-fractionation by 2-dimensional agarose gel electrophoresis (Yadav et al. 2014), the recovered 1-3 kb cDNA fractions were PCR-amplified (26 cycles using the Mint-2 cDNA synthesis kit) and cloned downstream of the *S. cerevisiae* PMA1 promoter in the pDR196 yeast–*Escherichia coli* shuttle vector (Rentsch et al. 1995) into which a kanamycin resistance gene and *Sfi*A and *Sfi*B cloning sites had been introduced. For each soil cDNA library, recombinant plasmids were introduced into electro-competent *E. coli* cells (MegaX DH10B™ T1R Electrocomp™ cells, Invitrogen, Thermo Fisher Scientific, Illkirch, France) and at least 2 10⁶ kanamycin resistant bacterial colonies growing on agar medium were collected and pooled to constitute two soil cDNA plasmid libraries after alkaline lysis extraction (Sambrook and Russell 2001).

Yeast strains, media, and culture methods

Environmental cDNAs encoding sugar transporters were selected for their capacity to restore growth on mannose or xylose of yeast mutant strains deleted of their monosaccharide transporters. For mannose, we used the EBY.VW4000 *S. cerevisiae* mutant strain (*MATa leu2-3, 112 ura3-52 trp1-289 his3-Δ1 MAL2-8c SUC2 Δhxt1-17 Δgal2 stlΔ::loxP agt1Δ::loxP mph2Δ::loxP mph3Δ::loxP*) unable to use mannose, but also glucose, fructose and galactose as carbon sources (Wieczorke et al. 1999). For xylose, we used the EBY.X1-2 *S. cerevisiae*

strain which is the EBY.VW4000 strain in which had been introduced plasmids p414-TEF.X1 and p25G.X2 containing the *XYL1* and *XYL2* genes from *Scheffersomyces stipitis*, respectively (Young et al. 2011). These two genes code for a xylose reductase (*XYL1*) and a xylitol dehydrogenase (*XYL2*), which are the two enzymes activities, absent from *S. cerevisiae*, needed for the use of xylose as a carbon source. The EBY.VW4000 strain was grown at 30°C in liquid or on solid yeast nitrogen base medium (YNB, 6.7 g.l⁻¹ without amino acids, pH 5.9) supplemented with (in g.l⁻¹) adenine, 0.01; arginine, histidine and methionine, 0.02; lysine, tryptophan and uracil, 0.04; threonine and tyrosine, 0.05; isoleucine, leucine and phenylalanine, 0.06; valine, 0.15 and maltose, 20. Selection of EBY.VW4000 transformants expressing monosaccharide transporters was performed on solid YNB medium lacking uracil and supplemented with amino acids and mannose as sole carbon source. Concerning the EBY.X1-2 strain, it was grown at 30°C in liquid or on solid YNB medium lacking leucine and tryptophan and supplemented with 20 g.l⁻¹ maltose. Selection of EBY.X1-2 transformants expressing xylose transporters was performed on solid YNB medium lacking uracil, leucine and tryptophan and supplemented with xylose as sole carbon source. The wild-type *S. cerevisiae* CEN.PK2-1C and its mutant derivatives (EBY.VW4000 and EBY.X1-2) were complemented with the empty pDR196-*SfiIA/B* plasmid to use them as positive and negative controls during growth rate measurements on different monosaccharides, respectively.

Selection of fungal sugar transporters by functional yeast complementation

Both soil cDNA plasmid libraries (from spruce and beech forest soils) were used to transform *S. cerevisiae* using a standard lithium acetate protocol (Rose and Broach 1990). Concerning the functional screening on mannose, EBY.VW4000 transformed yeast cells were selected directly on a solid yeast nitrogen base (YNB) minimal medium supplemented with mannose (0.2 or 0.5%) and amino acids but lacking uracil. In the case of xylose-transporter screening, EBY.X1-2 transformed yeast cells were first plated on a solid YNB minimal medium

supplemented with 2% maltose and amino acids but lacking uracil, leucine and tryptophan. Then, after 3 days of growth, uracil prototrophic yeast colonies were collected, washed by centrifugation and resuspended in sterile water before being plated on solid YNB minimal medium supplemented with 2% xylose and amino acids but lacking uracil, leucine and tryptophan. Plasmids from the yeast transformants were extracted using the Zymoprep Yeast Plasmid Miniprep I (Zymo Research, Irvine, CA, U.S.A.) and used to transform One Shot TOP10 chemically competent *E. coli* (Invitrogen) to kanamycin resistance. After plasmid extraction (NucleoSpin Plasmid kit; Macherey Nagel, Hoerdt, France), inserts were Sanger sequenced using primer PMA (5'-CTCTCTTTTATACACACATTC-3') and additional internal primers. The different fungal sugar transporters retrieved from spruce and beech soil cDNA libraries were annotated spS1 to spS12 and spB1 to spB7, respectively. Both yeast mutant strains (EBY.VW4000 and EBY.X1-2) were further transformed with all of these different environmental fungal sugar porters to study their capacity to transport different C6 and C5 monosaccharides. Sequences of environmental transporters that support the findings of this study have been submitted to the EMBL database with accession codes LR862110-LR862128.

Phenotypic characterization of yeast transformants and growth rate measurements

The monosaccharide utilization profile of EBY.VW4000 transformants was performed using drop-test assays on solid YNB minimal medium (Rose and Broach 1990) and electrophysiology assays (Damon et al. 2011). Only mannose, fructose and glucose could be tested using drop-test assays as other sugar sources are either not assimilated or naturally transported by the EBY.VW4000 mutant strain. Due to the complexity of the methodological approach, electrophysiological analyses were only performed with potential mannose specific transporters (spS1 and spS2), found by screening of EBY.VW4000 cells, to test the transport of sugar constitutive of cellulose and hemicelluloses (cellobiose, glucuronic acid, glucose,

fucose, galactose, rhamnose, arabinose, mannose, fructose and xylose). Additionally, maltose
 and two disaccharides abundantly found in plant tissues (mannobiose and sucrose) were also
 tested using these electrophysiological assays. Procedures used to characterize by voltage-
 clamp recordings the sugar transporters spS1 and spS2 expressed in *Xenopus laevis* oocytes
 are detailed in Online Resource 1.

Growth rates of the transformed EBY.VW4000 yeast strains were measured in triplicate using
 a Bioscreen C system (Growth Curves USA, Piscataway, NJ) equipped with a wide-band
 filter (420 to 580 nm) recommended for optical density (OD) measurements (Young et al.
 2011). A 1 µl inoculum of fully-grown cultures diluted to $5 \cdot 10^6$ cells.ml⁻¹, was added to each
 well filled with 250 µl of YNB minimal medium supplemented with amino acids but lacking
 uracil and with 0.1%, 0.5% or 1% (w/v) of carbon source (either maltose, D-glucose, D-
 fructose, D-mannose or D-galactose). The experiment was run 4 days at 30°C using a medium
 amplitude and normal intermittent shaking (10s) and OD measurement every 10 min.

Inhibition of mannose transport by other non-assimilated carbon sources (either glucose or
 fructose) was assessed in media containing 0, 0.1, 0.5 or 1% mannose and supplemented with
 either 0 or 1% of either glucose or fructose. Growth rates of EBY.X1-2 transformants in
 presence of xylose as carbon source were tested in liquid YNB minimal medium
 supplemented with 1% xylose as described by Colabardini et al. (2014). Yeast transformants
 were first grown on plates containing solid YNB minimal medium supplemented with 2%
 xylose. After 21 days of incubation at 30°C, colonies were transferred in 3 ml of YNB
 minimal medium supplemented with 1% maltose. After 24 h of culture at 180 rpm and 30°C,
 1.5 10⁶ yeast cells were washed with water and transferred in 3 ml of YNB minimal medium
 supplemented with 1% xylose. Yeast-cell growth was estimated once every 2-3 days during a
 period of 21 days by measuring the absorption at 600 nm.

Exponential growth rates were analyzed using R (version 3.5.3) and the package Growthcurver (Sprouffske et al. 2016). Growth data were fitted to a standard logistic equation and the Area Under the Curve (AUC) parameter was estimated with a 95% confidence interval to be able to detect any significant change between each condition (P value < 0.05).

Phylogenetic analyses

Environmental transporter sequences were compared to closest reference sequences retrieved from public databases as well as to all sequences of fungal sugar transporters that have been functionally characterized. Sequences highly similar to the selected environmental sugar transporters were identified by BLASTp searches in the GenBank database. For the taxonomic assignation of environmental sequences, the ten best BLAST hits were selected. Amino acid sequences were aligned using MUSCLE (Edgar 2004). Maximum-likelihood (ML) phylogenetic trees were generated with PhyML v.3.0 (Guindon et al. 2010) using the LG substitution model (Le and Gascuel 2008) as implemented in SeaView v.4 (Gouy et al. 2010). Robustness of tree topologies was assessed by bootstrap analysis (1000 replicates). Tree was drawn using FigTree v.1.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results

Phylogenetic and functional diversity of characterized fungal sugar porters (SPs)

From more than 70 literature references, we identified 123 fungal sugar transporters, belonging to the SP family (TC 2.A.1.1) that had been functionally characterized (Online Resource 2). A majority (109) of these genes originated from 30 *Ascomycota* species with an overrepresentation of *Saccharomycotina* yeasts (15 species), among which *S. cerevisiae* is represented by 22 genes. The database included nevertheless two transporters from the *Glomeromycotina* (2 species) and 12 from the *Basidiomycota* (7 species).

In a majority of cases functional characterization consisted in the expression of the corresponding fungal genes in a *S. cerevisiae* mutant deleted of its endogenous transporter genes followed by growth tests of the corresponding recombinant strain on one or several sugars that could no longer be assimilated by the yeast mutant (Young et al. 2011; Online Resource 2). Based on this often-fragmentary functional information in terms of number and diversity of tested substrates, we classified the transporters as monosaccharide (M) or oligosaccharide (O) transporters. Monosaccharide transporters encompassed those capable of transporting C5 or C6 monosaccharides but also structurally related molecules such as polyols (e.g. glycerol, mannitol, *myo*-inositol or sorbitol), gluconic, galacturonic or quinic acids. Oligosaccharides tested as potential substrates were mostly disaccharides (e.g. sucrose, cellobiose, xylobiose, maltose or lactose) and more rarely tri- (e.g. cello- or maltotriose) or tetraoses (cellotetraose). In five cases, transporters had been shown to transport both mono- and oligosaccharides (MO transporters). This is the case of the *Kluyveromyces lactis* *LAC12* that transports both lactose and galactose (Riley et al. 1987; Baruffini et al. 2006), but also of the *AGT1*, *MPH2* and *MPH3* “maltose transporters” of *S. cerevisiae* whose deletions were needed to suppress monosaccharide transport in the EBY.VW4000 mutant strain (Wieczorke et al. 1999). Besides their substrate (sugars) specificities, fungal SPs have also been shown to

function as either facilitators (e.g. Jordan et al. 2016) or H⁺ symporters (e.g. Dos Reis et al. 2013) co-transporting a proton along with a sugar molecule. Since the transport mode was very rarely assessed for published transporters, this information was not analyzed further.

Despite the fact that almost all "M" transporters had not been tested on oligosaccharides and inversely regarding "O" transporters on monosaccharides (Online Resource 2), this simple classification was well supported in a global phylogenetic analysis of the characterized transporter sequences that grouped almost all "O" and "M" transporters in distinct and well-supported clades (bootstrap values >80%; Fig. 1). The very few exceptions to this rule may require additional functional data to further refine their true substrate specificity prior to speculate about occasional reversal of "O" transporters to transport monosaccharides. At least in the case of monosaccharide "M" transporters, sequences originating from a single species, belonging to *Ascomycota* (e.g. the *Saccharomycotina* yeast *Yarrowia lipolytica* or the *Pezizomycotina* *A. nidulans*) or to *Basidiomycota* (e.g. the *Agaricomycotina* *Laccaria bicolor*) were often distributed in different clades suggesting that divergences between these sequences occurred at least prior to the divergence between *Ascomycota* and *Basidiomycota*. Besides specialization towards mono or oligosaccharides, functional data available for most transporters (Online Resource 2) are however insufficient to attribute a more specific specialization in terms of transported substrates to most clades identified in the global phylogenetic tree. Nevertheless, we observed that six of the seven *myo*-inositol transporters included in the tree, originating from three *Ascomycota* and one *Basidiomycota* species respectively, grouped together to form a homogeneous clade (clade VII, Fig. 1). This is also the case for (i) four transporters, from four filamentous *Ascomycota* species, known to transport charged, acidic molecules, either quinic acid, galacturonic acid or 4-deoxy-L-erythro-5-hexoseulose uronate, a constituent of alginate (clade IX) or (ii) two glycerol transporters from the yeasts *S. cerevisiae* and *Debaryomyces hansenii* (clade XI).

Regarding monosaccharide transporters, 52 of them had been tested on at least the four C6 sugars D-glucose, D-galactose, D-mannose and D-fructose that share the same molecular formula ($C_6H_{12}O_6$). While 25 of them (48%) transported all four sugars and 42 of them (81%) glucose, only five of them were apparently specific for one of these four sugars, three for fructose, one for mannose, one for galactose and none for glucose (Online Resource 2).

Regarding xylose transporters, widely studied in the context of plant biomass utilization, of the 24 xylose transporters studied for their capacity to transport at least one C6 monosaccharide, only one of them (*XltB* from *A. niger* tested on mannose, glucose, galactose and fructose) was seemingly specific for xylose (Sloothaak et al. 2016). This analysis suggests that strict specificity towards one of the most common sugar constitutive of plant biomass is a rare, if not inexistent, feature among fungal SPs.

Focusing on the transport of mannose, we observed a close association between mannose and glucose transport (Online Resource 2). Indeed, of the 47 published mannose transporters tested on glucose, only two (both from the yeast *Y. lipolytica*) could not transport glucose. Conversely, of the 49 published glucose transporters tested on mannose, only five (from *S. cerevisiae*, *Neurospora crassa* and *Piriformospora indica*) could not transport mannose.

Phylogenetic diversity of sugar transporters isolated from soil cDNA libraries

The screening of two forest soil metatranscriptomic cDNA libraries for the functional complementation of the growth defect of the EBYVW4000 yeast mutant on mannose-containing media led to the isolation of 15 different cDNAs; 11 from the spruce (*P. abies*, spS genes and proteins) library and 4 from the beech (*F. sylvatica*, spB genes and proteins) one. A similar screening on xylose-containing media using the same yeast strain expressing the *S. stipitis* xylose catabolic pathway led to the isolation of 4 cDNAs; 1 from the spruce library and 4 from the beech one. Eighteen of these 19 transporters were unambiguously attributed to

the SP family (TCDB #2.A.1.1). In blast searches against the curated SwissProt or TCDB databases, their best hits all belonged to this family and they were all predicted to have 12 transmembrane domains that displayed characteristic signature sequences for this SP family (Online Resource 3). Conversely, the spB4 transporter, that also possessed 12 putative transmembrane domains, displayed high similarity values to members of the so-called “Fucose: H⁺ Symporter” (FHS) family (TC 2.A.1.7). Members of this FHS protein family have been experimentally shown to transport different monosaccharides in *Bacteria*, including L-fucose in the case of the *E. coli FucP* transporter (Gunn et al. 1994). Although members of this family have been predicted in the proteomes of many (but not all) fully sequenced fungal species, only the *RhtA* gene from *A. niger* has been demonstrated to encode a L-rhamnose transporter (Sloothaak et al. 2016).

A phylogenetic analysis that incorporated all 19 transporters and their 10 most similar sequences identified in blast searches against the generalist GenBank nr database suggested that all these 19 environmental sequences most likely originated from *Fungi*. Ten of them could originate from *Ascomycota* and eight from *Basidiomycota* (Fig. 2). Regarding the divergent spB4, although most likely of fungal origin, its exact taxonomic placement could not be precisely ascertained as similar sequences originated from *Basidiomycota* but also *Chytridiomycota* (namely *Gonapodya prolifera*, belonging to the *Monoblepharidomycetes*; Fig. 2). BLASTp searches for spB4 homologs in fully sequenced fungal genomes showed that homologous sequences were absent in numerous fungal species and extremely rare in specific taxonomic groups such as the *Saccharomycotina* yeasts where a single sequence was identified in the genome of *S. stipitis*. A preliminary phylogenetic analysis using sequences from different fungal phyla, as well as from *Bacteria*, placed the spB4 environmental sequence, with other sequences from *Chytridiomycota*, *Ascomycota* and *Basidiomycota* in a clade (Online Resource 4) associated to a second clade encompassing bacterial sequences, as

well as a sequence from the *Mucoromycotina Mucor circinelloides*. Sequences identified in the genomes of fungal species extensively studied for sugar transport (*Aspergillus* sp., *N. crassa*, *Trichoderma reesei*, *S. stipitis*, but also *Tuber melanosporum*; Online Resource 1), including the *RhtA* rhamnose transporter from *A. niger*, grouped together to form another distinct clade (Online Resource 4).

When all environmental sequences were incorporated in the phylogenetic tree that encompassed nearly all characterized fungal SPs, we observed that all of them (except the divergent spB4 sequence used to root the tree) fell in clades of monosaccharide "M" transporters (Fig. 1). Furthermore, more than half (11) of the environmental sequences fell in a single clade (clade III, Fig. 1) that encompassed exclusively characterized proteins from *Ascomycota* species although five of the environmental sequences attributed to this clade were predicted to be from *Basidiomycota* (spS1, S2, S7, B2 and B7; Fig. 2).

Monosaccharide utilization profiles of sugar transporters isolated from soil cDNA libraries

Substrate range and preferences for D-monosaccharides was assessed by measuring the growth of the recombinant EBYVW4000 yeast strain constitutively expressing each of the environmental transporters on different concentrations of either fructose, galactose, glucose and mannose as the sole C sources in both liquid (Fig. 3) and solid (Online Resource 5) media. Additionally, growth of the recombinant EBY.X1-2 yeast strain expressing each of the environmental transporters in combination with the *S. stipitis* xylose assimilation pathway was assessed on a medium containing 10 g.l⁻¹ of xylose as sole carbon source (Online Resource 6). Regarding the use of C6 sugars a great variability was observed among environmental transporters. While for each transporter, mannose was always the best sugar, or among the best, in terms of final biomass yield at the end of the culture period (4 days), this yield was particularly low for several transporters (namely spB4, spS2 and spS7) on all of the tested concentrations of the different sugars. This may be the result of improper expression of

these proteins in yeast (as already reported for sugar transporters by e.g. Dreyer et al. 1999; Wieczorke et al. 2003) or may suggest that in vivo, in their respective fungal species, these transporters may preferentially transport other sugars.

While several transporters could be categorized as relatively generalists (e.g. spB2, spS4, spS8, spS9 or spS11), leading to comparable growth rates on all four C6 sugars tested, others had a more restricted substrate range. Noticeably, five environmental transporters (spB3, spB4, spS1, spS2 and spS7) did not allow growth of the recombinant yeasts on all three tested concentrations of glucose (particularly well visible in drop tests on solid media, Online Resource 5). This proportion of "glucose negative" environmental transporters among "mannose positive" ones (26%) is significantly higher (Chi2 test, $P < 0.01$) than the proportion observed among published transporters (two in 47; 4%). Among these five SPs, the phylogenetically related spS2 and spS7 ones (Fig. 2) were seemingly mannose specific in both liquid and solid cultures. However, both spS2 and spS7 were among the transporters for which the final biomass yield was always low irrespective of the monosaccharide added to the culture medium. Altogether, clustering of environmental transporters according to their substrate range, deduced from the growth of the recombinant EBYVW4000 strain on different concentrations of the four C6 monosaccharides (Fig. 3) demonstrated an absence of correspondence between substrate preferences and phylogenetic proximity of the primary sequences (Fig. 1 and 2).

As already reported in the literature, growth on xylose of recombinant yeast strains expressing a heterologous xylose catabolic pathway is intrinsically low (Tran Nguyen Hoang et al. 2018). We could nevertheless show that 18 out of the 19 environmental transporters supported growth of this strain on xylose (Online Resource 6). Furthermore, all of these proteins harbored the amino-acid motif "G-G/F-XXX-G" (where X represents a variable, but usually non-polar, amino acid residue) in the first predicted transmembrane domain (Online

Resource 3). According to Young et al. (2014), this motif represents a signature for xylose transport in SPs. Only the transporter spB4 belonging to FHS family did not support xylose assimilation at all and did not present this xylose specific amino-acid motif (Online Resource 3).

To further analyze substrate range and specificity of the environmental transporters, we hypothesized that they could function as symporters and two of them (spS1 and spS2 featuring different substrate specificities in growth tests) were expressed in *X. laevis* oocytes to measure electrophysiological responses in presence of mono- or disaccharides including several sources (e.g. L-C5 or L-C6 monosaccharides, cellobiose or mannobiose) that are not assimilated by the wild-type *S. cerevisiae*. The selected transporters were spS1, which supported good growth on mannose, moderate growth on fructose, galactose and xylose and no growth on glucose and spS2 supporting poor growth on all tested C6 sugars (Fig. 3). Transport of mannose was confirmed for both selected transporters as expected from the yeast complementation assay (spS1, n=24, N=7; spS2 n=16, N=7; where n is the number of studied oocytes from N different batches) in test solutions complemented with 10 mM mannose at pH 5.5 (Fig. 4). When applying hyperpolarizing voltage pulses, negative currents of up to several 100 nA were induced dependent on the applied potential, thus indicating that both spS1 and spS2 indeed functioned as sugar/ion co-transporters (Fig. 4A,B). Current induction by mannose was not observed in water-injected control oocytes (n=4, N=3) and was reversible in transporter-expressing oocytes, as perfusion with control solution reduced the substrate specific currents (reversibility was tested for all recorded currents). These sugar-specific negative currents would correspond to cation influx (or anion efflux). The observed currents could be shown to be dependent on external pH (i.e. H^+ concentration) as no currents were induced with mannose at pH 7.5 (n=3, N=2; Fig. 4C) indicating influx of protons and thus H^+ -mannose symport. Several sugar transporters have been described to function as H^+ -sugar

symporters (Bush 1990; 1993; Wolf et al. 1991; Boorer et al. 1996; Lemoine et al. 1996; Zhou
 et al. 1997; Rottmann et al. 2016). Amplitudes of current induction varied between oocytes
 probably because of different expression levels in oocytes and oocyte batches. Interestingly,
 both transporters differed clearly with respect to transport of glucose (Fig. 4C). In agreement
 with growth measurements, the transporter spS1 did not transport glucose (n=10, N=4;
 Fig. 4C) whereas spS2 mediated undoubtedly glucose transport (n=6, N=5; Fig. 4B,C). In
 contrast, fructose was never transported by spS2 (n=6, N=5; Fig. 4C) but partly by spS1 (n=5,
 N=3; Fig. 4A,C). However, first experiments suggested that spS1 did not transport fructose
 (n=4, N=3) most probably by an inhibitory effect of the non-transported glucose due to the
 sequential arrangement of the tests. This result suggests that glucose might bind to the spS1
 transporter without inducing currents, thus hindering transport of other substrates. The
 observed differences for the transport of glucose and fructose for the two tested mannose
 transporters spS1 and spS2 underline the specificity of such transport systems. Interestingly,
 both mannose transporters mediated currents in oocytes only for D-mannose but not for L-
 mannose (Fig. 4C; spS1 n=3, spS2 n=4) pointing to highly specific structural requirements
 needed for binding or transport. Further tests of substrates with either D- or L-configuration
 were pooled and confirmed the structural substrate specificity. The “L-pool” with L-
 arabinose, L-rhamnose, and L-fucose did not induce any current (spS1 n=5, spS2 n=1). The
 “D-pool” with D-galactose, D-xylose, and D-glucuronic acid provoked unfortunately
 endogenous currents also in control oocytes, so that these three substrates had to be tested
 separately. In fact, D-glucuronic acid destabilized oocytes, even control ones, and could not
 be tested. However, in agreement with growth measurements, D-galactose and D-xylose
 induced currents for both transporters (n=3, each; Fig. 4C). In agreement with the
 phylogenetic placement of the spS1 and S1 transporters among monosaccharide (M)
 transporters (Fig. 1), four tested disaccharides (maltose (n=5, each), sucrose (spS1 n=3, spS2

n=4), mannobiose (n=4 each), and cellobiose (n=4 each)) did not activate any currents in oocytes expressing the two transporters.

Glucose inhibition of transport of other monosaccharides by SPs that do not transport glucose, as suggested here in the oocyte experiment for spS1, has been repeatedly reported in the literature (Subtil and Boles 2012; Farwick et al. 2014). We evaluated this phenomenon, as well as potential inhibition by fructose, for five environmental transporters (spS1, spS2, spS7, spB3 and spB6) that did not support significant growth on any concentration of both glucose and fructose. For all five cases, we observed that in the presence of 10 g.l⁻¹ of either glucose or fructose in the culture medium, the EBYVW4000 strain expressing the corresponding transporters failed to grow on either low (1 g.l⁻¹), medium (5 g.l⁻¹) or high (10 g.l⁻¹) concentrations of mannose (Online Resource 7).

Discussion

The compilation of possibly all fungal SPs characterized thus far and their inclusion in a phylogenetic context support earlier observations, made on more fragmentary datasets, of a clear separation between mono- and oligosaccharide SPs in the Fungi (e.g. Peng et al. 2018). However, the present analysis did not address the issue of potentially multiple origins of either mono- or oligosaccharide clades of SPs in the *Fungi*. The presence, in several of the clades, of transporters from both the *Basidiomycota* and *Ascomycota* and even from the *Glomeromycotina* suggests that the corresponding clades originated early during the diversification of the *Fungi*. Alternatively, this distribution of transporters across the phylogenetic tree could also be the results of horizontal gene transfers between species belonging to different clades as demonstrated for other transporter genes (Slot et al. 2007; Coelho et al. 2013).

Confrontation of the functional database to the SP phylogenetic tree suggested that few clades of "monosaccharide transporters" may feature functional specialization on specific substrates structurally related to monosaccharides, such as *myo*-inositol, acidic sugar derivatives or glycerol. This phylogenetic information could thus be used for the functional annotation of similar transporters from sequenced fungal genomes. As for the other clades of monosaccharide transporters, they encompass sequences that transport bona fide monosaccharides (either C5 or C6 sugars), but also sugar alcohols without any clear correspondence between clade identity and the exact nature of the transported molecules. While for several yeast species (e.g. *S. cerevisiae* or *Schizosaccharomyces pombe*), most of their monosaccharide SP genes seemed to originate from recent duplication events and belong to the same clade, other species (e.g. *A. nidulans*, *N. crassa*, or *Y. lipolytica*) seem to rely on different highly divergent SPs, belonging to different clades for their supply of monosaccharides.

A comprehensive analysis of the substrate range of monosaccharide transporters indicates that a large majority of the studied transporters accepts several sugars as substrates and that specificity towards one or two molecules seems to be an exception (Online Resource 1). In this respect, occasional claims of a substrate-specific transporter often reflect the fact that a very limited number of potential substrates have been studied (e.g. Wang et al. 2016). It could however be argued that overexpression of a sugar transporter in the yeast *S. cerevisiae*, the most widely used approach to characterize such transporters from fungi (Young et al. 2011), may overestimate the substrate range of the corresponding transporter. Overexpression may indeed allow significant influx of otherwise poorly transported substrates (Dreyer et al. 1999) that would not normally support cell multiplication. These experimental artifacts can be partly solved using the more labor-intensive expression of transporters in *X. laevis* oocytes for electrophysiological measurements that also allows the evaluation of molecules that are not normally assimilated by *S. cerevisiae* (e.g. C5 or L-sugars) as potential substrates. Using this approach with two environmental SPs originally selected on mannose, we confirmed yeast growth assay observations by showing that both spS1 and spS2 transport mannose, xylose and galactose, and that they differ in their specificity towards fructose and glucose. Corresponding to the yeast assays, we also observed that one of them accepted fructose, the other one glucose as a substrate. Furthermore, we demonstrated that both transporters did not accept L-monosaccharides and di-saccharides as substrates.

Although not being specific towards one or two potential substrates, many transporters are neither truly generalists and clearly do not transport several "common" monosaccharides. Overrepresentation of glucose-transporting monosaccharide transporters may reflect the widespread occurrence of this sugar in nature (albeit often as monomer of polysaccharides) and its centrality in carbon metabolism (Gunina and Kuzyakov 2015). Alternatively, it could also be hypothesized that overrepresentation of glucose-transporting transporters in databases

may reflect a scientific bias as numerous fungal transporters were precisely selected for their capacity to transport glucose. For the specific case of mannose, the high prevalence of glucose-transporting porters that can also transport mannose could be explained by the observation that, at least in yeast, genes encoding enzymes necessary for its use as a carbon source (hexokinases and mannose-6-phosphate isomerases) are expressed in both glucose and mannose-containing media. This suggests that these two sugars can be assimilated simultaneously, with however a preference for glucose over mannose.

By selecting transporters from metatranscriptomes based on their capacity to transport mannose, we tested the hypothesis that "mannose specific" transporters, or at least transporters that preferentially transport mannose over glucose may be present in soil organisms occasionally confronted to carbon sources with particularly high mannose content (e.g. gymnosperm plant biomass or fungal necromass) (Lipke and Ovalle 1998; Schädel et al. 2010)). As previously observed in the case of oligopeptides (Damon et al. 2011), the screening of environmental cDNA libraries for sugar transporters yielded genes from only *Fungi*, although genes from other eukaryotic organisms were present among the studied metatranscriptomes (Damon et al. 2012). Furthermore, all but one of the selected genes encoded proteins that belonged to the SP family, suggesting that other sugar transporters reported in fungal genomes may play a minor role in sugar assimilation by fungi. From a taxonomic perspective, eight of the environmental transporters likely originated from *Basidiomycota*, thus doubling the number of characterized sugar transporters from this fungal phylum published thus far (Online Resource 1). From a phylogenetic perspective, environmental SPs were distributed in several of the distinct clades that form this protein family. However, more than half of them (11), from both *Basidiomycota* and *Ascomycota*, fell in a single clade (clade III in Fig. 1) that, thus far, encompassed only published *Ascomycota* sequences. This uneven distribution of environmental SPs across the global phylogenetic tree

of this fungal protein family could originate from a preferential functional expression of these
 structurally related proteins in yeast. It could also suggest that *Fungi* preferentially use SPs
 from a limited number of clades for their supply in monosaccharides. This latter hypothesis
 could be addressed in model organisms by measuring the relative expression levels and
 contribution to monosaccharide utilization of endogenous sugar porter encoding genes
 belonging to different evolutionary lineages. Finally, screening of soil metatranscriptomes
 also yielded a highly divergent fungal sugar transporter sequence (spB4) that belonged to the
 poorly studied “Fucose: H⁺ Symporter” (FHS) family (TC 2.A.1.7). In *Fungi*, only one
 member of this FHS family able to transport monosaccharides was reported so far. In *A.*
niger, the related *RhtA* transports L-rhamnose and marginally D-fructose (Sloothaak et al.
 2016). Since both rhamnose and fucose are deoxy sugars that occur in nature in the unusual
 levorotary (L-) conformation, it could be hypothesized that fungal FHS family members are
 specialized on these substrates and marginally accept other sugars as in the case of spB4 that
 confers poor growth of recombinant *S. cerevisiae* on mannose and all other tested sugars. This
 hypothesis could be tested by expression of this protein, as well as other homologous ones
 found in fungal genomes, in *X. laevis* oocytes as performed for the spS1 and spS2 SPs.
 Overall, our observations that result from the analysis of "environmental" sequences further
 illustrate the power of functional genomics for quickly accessing the true diversity of specific
 eukaryotic protein families (Damon et al. 2011; Marmeisse et al. 2017).

From a functional perspective, the analysis of environmental fungal sequences
 reevaluates the known functional diversity of fungal SPs. It revealed the existence of
 phylogenetically distinct transporters that allow mannose, but not glucose transport. This
 lends support to the hypothesis that the presence of sugars other than glucose in the
 environment may have led to the independent evolution of sugar transporters that
 preferentially use mannose (and eventually other monosaccharides) over glucose.

Furthermore, as an excess of glucose seemingly inhibits these transporters, this observation further suggests that indeed they may only be operational in environments depleted of this sugar.

"Other than glucose" (e.g. xylose) monosaccharide transporters have been searched for the independent, simultaneous and non-competitive entry of glucose and other sugars in fungal (e.g. yeast) cells for their simultaneous co-fermentation. Alternatively, these transporters can also be used for the specific utilization of the "other than glucose" monosaccharide as carbon source in a context where glucose is also present. One limitation to this strategy is that transporters that do not transport glucose are often inhibited by this sugar (Farwick et al. 2014; Li et al. 2016) which, possibly, clogs the entry pore of the protein. We showed that this phenomenon may also affect mannose transport by mannose porters that do not support glucose transport. Furthermore, we also show that this phenomenon is not glucose-specific but can also be generated by other non-transported sugars such as fructose. Several of the selected environmental transporters could thus be used for the co-fermentation of mannose and other sugars or the specific use of mannose in the presence of other sugars, providing that their inhibition by competing substrates could be relieved (Farwick et al. 2014). In the case of xylose transporters, mutagenesis and directed evolution, which resulted in a limited number of changes in the primary sequence, have been successfully employed to render glucose-insensitive the corresponding transporters (Farwick et al. 2014; Li et al. 2016).

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Author contribution

FB, PL and RM conceived and designed research. FB, LV, CG-G, SDZ, DM, JD and PL conducted experiments. LF-T co-lead with PL the first part of the experiments performed by FB. CG-G and SDZ performed and interpreted electrophysiological measurements. ML contributed new reagents and analytical tools. FB, LV, SDZ, DM, DA, PL and RM analyzed data. SDZ, PL and RM wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest

Ethical approval

Experiments using oocytes from *Xenopus* frogs have been performed accordingly to the French national rules for animal maintenance and experiments (agreement A34172044).

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Legends of the Figures

Fig. 1 Phylogenetic diversity of 104 functionally characterized fungal sugar transporters

belonging to the Sugar Porter (SP) family. The maximum likelihood (PhyML) tree was rooted

using the environmental spB4 protein that belongs to the “Fucose H⁺ symporter” family.

Only bootstrap values (1000 replicates) above 80% are indicated and proteins were grouped in

13 clades (I to XIII). Colors refer to the taxonomic origin of the proteins, red, *Ascomycota*;

blue, *Basidiomycota* and black, *Glomeromycotina*. Proteins were named according to the

species they come from and the category of molecules they transport; M, monosaccharides

and related molecules; I, *myo*-inositol; O, di and oligosaccharides; S, sucrose; MO, both mono

and disaccharides. Full correspondence between protein names, species names, amino acid

sequences and accession numbers are given in Online Resource 3. Asterisks highlight

environmental sequences selected from soil cDNA libraries. For legibility, we excluded from

the final phylogenetic analysis several characterized sequences from the yeasts

Saccharomyces sp. that in preliminary analyses were all closely related to the sequences from

these species retained for the final analysis.

Fig. 2 Putative taxonomic origins of the 19 environmental sugar transporter proteins retrieved

from soil metatranscriptomic cDNA libraries. The phylogenetic (PhyML) tree encompasses

all 19 environmental sequences (asterisks) and their 10 best hits following BLASTp searches

in the GenBank database (144 reference sequences in total). Colors refer to the taxonomic

origin of the proteins, red, *Ascomycota*; blue, *Basidiomycota* and black, *Chytridiomycota*

(*Monoblepharidomycetes*). Only bootstrap values (1000 replicates) above 80% are indicated.

Fig. 3 Growth of recombinant *S. cerevisiae* EBYVW4000 strain expressing each of the 19

environmental transporter proteins (spS1-12; spB1-7) in liquid media containing 3

concentrations (1; 0.5 or 0.1%) of 4 D(+)-monosaccharides (glu, glucose; gal, galactose; fru,

fructose and man, mannose). “Area under the curves” were measured after four days’ growth

in microplates and used to draw a heatmap where sugar transporters clustered according to the growth profile of the recombinant yeast strains. Panels on the right illustrate the growth profiles on the different sugar concentrations (as well as on 1% maltose used as a positive growth control) for recombinant yeasts expressing 3 genes, each representative of one of the three main clusters observed in the heat map (spS1 for cluster 1, spS2 for cluster 2 and spS11 for cluster 3).

Fig. 4 Mannose sugar porters spS1 and spS2 induce sugar-specific pH-dependent currents when heterologously expressed in *Xenopus laevis* oocytes. (A,B) Representative examples of mannose-induced currents in oocytes expressing spS1 (A) or spS2 (B), respectively, recorded by two-electrode voltage-clamp in presence of 10 mM mannose at pH 5.5. To a similar but slightly minor extent, also other substrates are transported, as fructose by spS1 (A), or glucose by spS2 (B). (C) Mean current amplitudes induced by different sugar substrates in oocytes expressing spS1 or spS2. Current amplitudes induced by different substrates have been determined at -100 mV after subtracting initially endogenous background currents recorded in control solution without the added substrate. Mannose-induced currents measured at pH 5.5 (“mann” S1 n=24, N=7; S2 n=16, N=7) are shown to be pH-dependent as no current induction was observed at pH 7.5 (“mann 7,5” S1 and S2 n=3, N=2) and to be conformation-dependent (“Lmann” S1 n=3, S2 n=4). Glucose was transported by spS2 (“gluc” S1 n=10, N=4; S2 n=6, N=5), fructose by spS1 (“fruct” S1 n=5, N=3; S2 n=6, N=5). Galactose (“gal” S1 and S2 n=3) and xylose (“xyl” S1 and S2 n=3) induced currents by both transporters.

Fig. 2

Fig. 3

Fig. 4

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