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Genome-wide prediction and transcriptome analysis of sugar transporters in four ascomycete fungi

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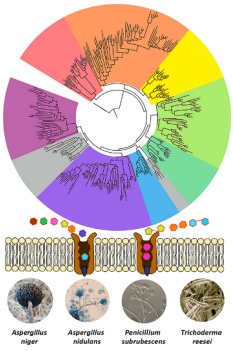
HIGHLIGHTS

- 342 sugar transporters (STs) were predicted from four ascomycetes.
- Transcriptomics analysis of STs on nine sugars showed a complex expression profile.
- Co-expression pattern indicated possible function for newly predicted STs.

GRAPHICAL ABSTRACT



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ABSTRACT

The import of plant-derived small sugars by sugar transporters (STs) has received increasing interest due to its important biological role and great industrial potential. STs are important targets of genetic engineering to improve fungal plant biomass conversion. Comparatively analysis of the genome-wide prevalence and transcriptomics of STs was performed in four filamentous fungi: *Aspergillus niger*, *Aspergillus nidulans*, *Penicillium subrubescens* and *Trichoderma reesei*. Using phylogenetic analysis and literature mining, their predicted STs were divided into ten subfamilies with putative sugar specificities assigned. In addition, transcriptome analysis revealed complex expression profiles among different STs subfamilies and fungal species, indicating a sophisticated transcriptome regulation and functional diversity of fungal STs. Several STs showed strong co-expression with other genes involved in sugar utilization, encoding CAZymes and sugar catabolic enzymes. This study provides new insights into the diversity of STs at the genomic/transcriptomic level, facilitating their biochemical characterization and metabolic engineering.

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1. Introduction

In nature, plant biomass is the major carbon source for most fungi. Fungal biotechnology has shown great potential in converting abundant, renewable plant biomass into valuable biofuels, biochemicals and food (Meyer et al., 2020). Therefore, a deep understanding of the related molecular mechanisms is crucial for advancing the transition from the current petroleum-based economy into a bio-based circular economy. Especially, metabolic engineering of sugar transporters (STs) has been demonstrated to be a promising strategy towards enhanced fungal sugar utilization in biotechnological applications (Leandro et al., 2009; Nogueira et al., 2020). For example, heterologous expression of pentose transporters or engineering of an endogenous hexose transporter have been performed in *S. cerevisiae* to enable it to co-consume both hexose and pentose sugars, which could facilitate biofuel production from plant lignocellulose materials (Nijland and Driessen, 2020).

The uptake of sugars across the cell membrane requires the assistance of STs. Most of the characterized fungal STs belong to the sugar porter (PFAM domain PF00083) family, a member of major facilitator superfamily (MFS, PFAM clan CL0015). Previous comparative genomics analysis and biochemical characterization have not only revealed biological functions of STs, but also provided new insights into exploring the fungal potential for industrial applications (Nogueira et al., 2020). For instance, a comparative genomics study has revealed the expansion of ST genes in Pezizomycotina fungi compared to Saccharomycotina, which could explain the different carbon source utilization approaches of these two fungal groups (Cornell et al., 2007). A good example is *Saccharomyces cerevisiae* which cannot metabolize pentose sugars and lacks the corresponding STs in its genome (Leandro et al., 2009). In contrast, hexose transporters (Hxt) have been expanded in *S. cerevisiae* to facilitate its adaptation to glucose-rich habitats (Lin and Li, 2011). The filamentous fungus *Aspergillus niger*, in turn, was predicted to contain 86 STs divided into nine subfamilies displaying complex expression and regulation, which could contribute to the broad habitats and high nutritional versatility of *A. niger* (Peng et al., 2018).

Fungal STs have shown notable functional redundancy and complexity. A fungal genome typically contains multiple STs with similar substrate specificity, as well as individual transporters with the ability to transport different sugars (Peng et al., 2018). For instance, three STs of *Penicillium oxalicum*, CdtC, CdtD and CdtG, have been shown to mediate the uptake of cellodextrin (Li et al., 2013) with different affinities and efficiencies. Similarly, multiple D-xylose transporters have been characterized in *A. niger* and *Trichoderma reesei* (Huang et al., 2015; Sloothaak et al., 2016b). On the other hand, one of the well-studied transporters in *A. niger*, MstA, is able to transport multiple sugars, e.g. D-glucose, D-fructose, D-xylose and D-mannose (vanKuyk et al., 2004).

Gene expression analysis has revealed a sophisticated regulation of specific STs. For instance, expression of *xltA* and *str1* are induced by D-xylose and dependent on the XlnR/Xyr1 regulators in *A. niger* and *T. reesei*, respectively (Sloothaak et al., 2016b), whereas the expression of *A. niger mstA* is controlled by CreA-mediated carbon catabolite repression and regulated by PacC in response to pH change (vanKuyk et al., 2004). Additionally, increasing evidence supports the function of several STs as transceptors, which are not only involved in sugar transport, but also play a role in sensing environmental sugar levels and triggering corresponding intracellular signaling (dos Reis et al., 2016). The deletion of ST genes has been shown to affect fungal growth and extracellular enzyme production (dos Reis et al., 2016; Havukainen et al., 2020; Huang et al., 2015; Zhang et al., 2013).

Although the investigation of individual STs in limited fungal species have importantly shed light on their biochemical features and physiological roles, more (functional) genomic studies are needed to better understand the diversity of STs across fungal species. In this study, a systematic investigation of the genomic and transcriptomic profiles of STs was performed in four diverse ascomycetes, *A. niger*, *Aspergillus nidulans*, *Penicillium subrubescens* and *T. reesei*. Among them, *A. niger*, *A. nidulans* and *T. reesei* are well-known industrial workhorses and model organisms for production and research of cellulases (Meyer et al., 2020), hemicellulases (Stricker et al., 2008) and other bioproducts, such as citric acid (Tong et al., 2019). In addition to the industrial strains, *P. subrubescens* is a potential producer of many plant biomass degrading enzymes (Mäkelä et al., 2016). The study revealed that the candidate STs encoded in these fungal genomes are highly variable in terms of numbers and their biochemical properties, and different fungi have selectively expanded or reduced specific ST subfamilies. Comparison of transcriptomes on a broad set of monosaccharides revealed diverse expression profiles across ST subfamilies and fungal species, suggesting the presence of complex regulation mechanisms. In addition, ST genes were co-expressed with other key genes involved in sugar utilization, such as Carbohydrate-Active Enzymes (CAZymes) and sugar catabolic enzymes (SCEs).

2. Materials and methods

2.1. Identification of sugar transporters

Through manual literature search, 174 experimentally validated fungal STs were collected (see supplementary materials). The majority (157) of these genes originated from Ascomycete species, including 14 genes from *A. nidulans*, 11 from *A. niger*, and 16 from *T. reesei*. Other known STs included one from Glomeromycota and 13 from Basidiomycota species. 171 STs containing the PFAM ST domain (PF00083) were selected for subsequent analysis.

The proteomes of *A. niger* NRRL3 (https://mycocosm.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html), *A. nidulans* FGSC A4 (https://mycocosm.jgi.doe.gov/Aspnid1/Aspnid1.home.html), *P. subrubescens* CBS132785 (https://mycocosm.jgi.doe.gov/Pensub1/Pensub1.home.html) and *T. reesei* QM6a (https://mycocosm.jgi.doe.gov/Trire_Chr/Trire_Chr.home.html) were downloaded from the JGI MycoCosm database. The ST domain (PF00083) profile obtained from the PFAM database was used to search against the sequences of the downloaded proteomes and collected known STs using the "hmmsearch" of the HMMER tool (Eddy, 1998). The lowest score (233.2) observed among the "hmmsearch" results of all the known ST was set as a cutoff. In addition, the predicted STs were further filtered based on annotation of transporter classification database (TCDB) (Saier Jr et al., 2006), and only the ST candidates with best blast hits in TCDB assigned to transporters of sugar, sugar alcohol or sugar acid were included for further analysis.

2.2. Phylogenetic analysis

Protein sequences were aligned with MAFFT (Katoh and Standley, 2013). The positions that contained > 20 % gaps were removed from alignment using trimAl (Capella-Gutiérrez et al., 2009). The phylogenetic analysis was performed with maximum likelihood method using IQ-TREE 2 (Minh et al., 2020) with 1000 UFBoot2 bootstrapping and MFP model. The other parameters were set as default, and seven STs from *Arabidopsis thaliana* (Büttner, 2010) were included as out-group in the phylogenetic analysis. iTOL (Letunic and Bork, 2016) was used to visualize the tree.

2.3. Identification of orthologs

Ortholog analysis was done with OrthoFinder (Emms and Kelly, 2019) using default parameters. For each ortholog group, the corresponding phylogenetic tree was manually inspected to exclude possible paralogs.

2.4. Transcriptome and co-expression analysis

Transcriptome data of *A. niger* NRRL3 was obtained from a recently

published study (Li et al., 2023). The RNA-seq data of other three species was published previously (Li et al., 2022). Briefly, mycelia of *A. niger*, *A. nidulans* and *P. subrubescens* were transferred to nine different monosaccharides for 2 h treatment before collection of the mycelia, while this period was 4 h for *T. reesei*. The gene expression level was measured as fragments per kilo-base of transcript per million mapped reads (FPKM) (Trapnell et al., 2010). The average values of replicates for each gene were log scaled and visualized on heatmaps using R package "pheatmap".

Co-expression between predicted STs and other plant biomass conversion (PBC) genes were conducted, including CAZymes, transcription factors (TFs) and SCEs. The expression values (FPKMs) of these genes were used to calculate the Pearson correlation coefficient (PCC). Co-expressed genes were defined as two genes with PCC > 0.9 and P-value < 0.05. The very lowly expressed genes (with maximum FPKM $<$ 10 in all tested conditions) were excluded from the co-expression analysis. The putative STs genes that were correlated with less than five other PBC genes were also excluded from further analysis. The co-expression network was visualized by R package "igraph" and "edgebundle". Enzyme classes of putative CAZymes were obtained from JGI MycoCosm database and their preferred substrates were manually added based on the specificity of characterized enzymes in same family or ortholog groups. The PBC related CAZymes and TFs were selected based on a previous study (Peng and de Vries, 2021) and ortholog

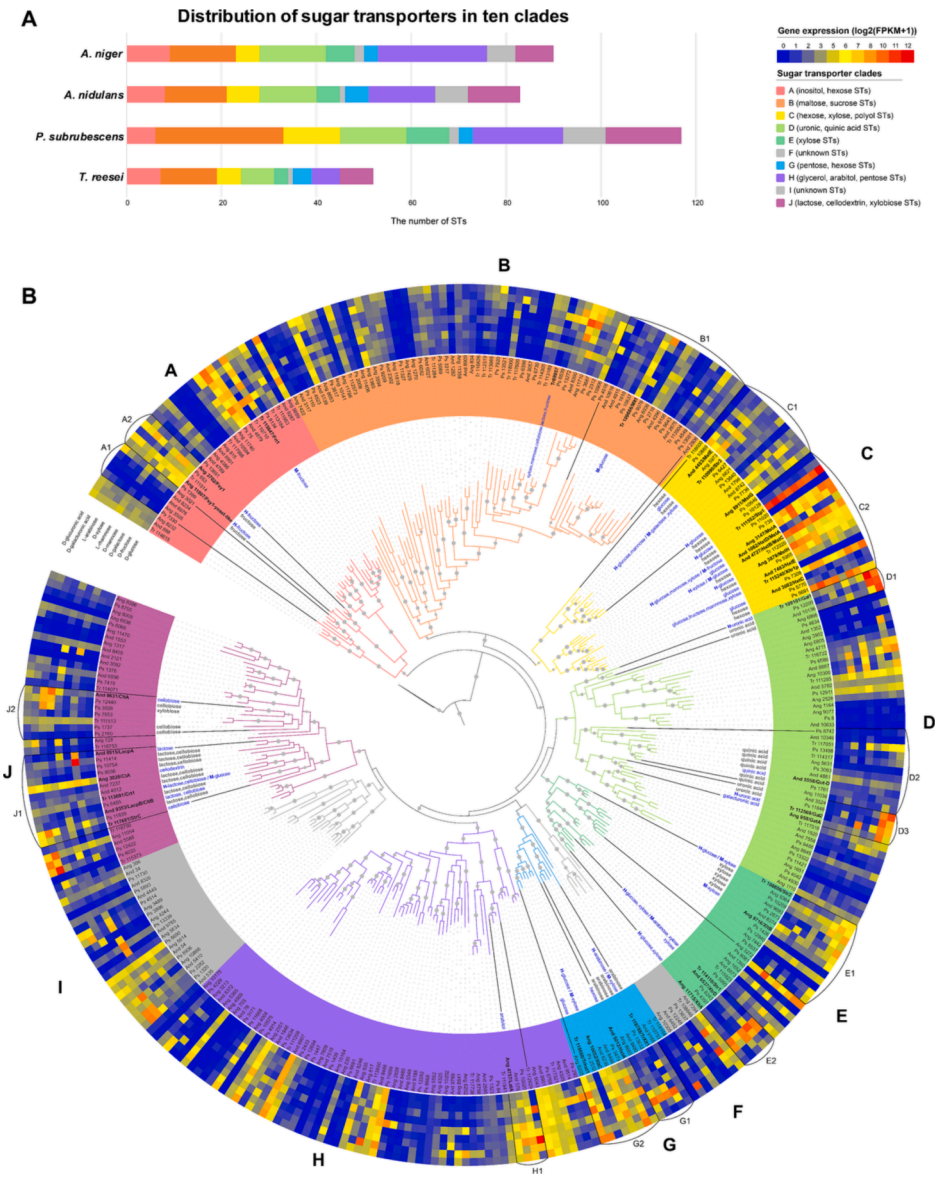

Fig. 1. Phylogenetic classification, distribution and expression profile of sugar transporters (STs) in *A. niger*, *A. nidulans*, *T. reesei* and *P. subrubescens*. (A) Distribution of sugar transporters in ten clades. (B) Phylogenetic tree and expression profile of predicted sugar transporters. The tree contains 342 predicted STs from four ascomycetes used in this study. Potential substrates for members of each clade were inferred based on the functions of the characterized transporters included in each specific clade. Average expression values (FPKMs) of each ST in each growth condition were plotted on the heatmap. JGI protein ID together with the abbreviation of species name (And = *Aspergillus nidulans*, Ang = *Aspergillus niger*, Ps = *Penicillium subrubescens*, Tr = *Trichoderma reesei*) are shown. Gene names of experimentally characterized STs are presented in bold font, of which STs with kinetic properties are marked with their affinity to specific sugars (H = high affinity, M = moderate affinity, L = low affinity) (see supplementary materials), and their defined function are in blue font. Putative functions of uncharacterized STs are in gray font. STs of *Arabidopsis thaliana* were used as an outgroup. Branches with bootstrap values > 60 % are indicated at the nodes with grey circles. The details of protein sequences are listed in supplementary materials. Different clades derived from phylogenetic analysis are highlighted in different colors. The subclades containing STs that were closely clustered with characterized STs on the phylogenetic tree are indicated with curved line in the outer circle of the figure.

analysis. The SCEs were derived from a recent study (Li et al., 2022).

3. Results and discussion

3.1. Phylogenetic profile of sugar transporters

Based on the domain search and manual inspection of the results on TCDB, 90, 83, 117 and 52 STs were obtained from *A. niger*, *A. nidulans*, *P. subrubescens* and *T. reesei*, respectively. The number of predicted STs for *A. niger, A. nidulans* and *T. reesei* is similar to previous studies (Chaudhary et al., 2016; Nogueira et al., 2020; Peng et al., 2018), and the sugar transportome of *P. subrubescens* was first examined.

Sugar specificity of predicted STs was assigned through incorporating the characterized STs in the phylogenetic tree based on the assumption that the genes of the same clade tend to share similar function (Eisen and Wu, 2002). In total, ten different clades named from A to J and supported with bootstrap values > 70 % were identified in the phylogenetic tree (Fig. 1B, see supplementary materials). The characterized STs are not equally distributed across different clades. Among them, clade C contains most of the characterized STs. Clade A, C, E, G and H were annotated as monosaccharide and polyol sugar transporters. Clade D contains uronic and quinic acid STs, while clade B consists of maltose, sucrose STs and clade J of lactose, cellodextrin, xylobiose STs. Clade F and I consist of uncharacterized sugar transporters.

The total number of predicted STs varies across the taxonomic distance of the studied fungi. The Eurotiomycete species have a similar number of total STs in their genomes, i.e., 90, 83, and 117 for *A. niger*, *A. nidulans* and *P. subrubescens*, respectively, while only 52 STs were identified for *T. reesei* from Sordariomycetes. The predicted STs in the studied species are not equally distributed across different clades (Fig. 1A). The two *Aspergillus* species have similar numbers of STs for each clade, except that *A. niger* has more STs in clade H and less in clade J (23 and 8, respectively) compared to *A. nidulans* (14 and 11, respectively). *T. reesei* has significantly less STs in clade D, E, H and I (7, 3, 6 and 0, respectively) than the other three species, while *P. subrubescens* contains more STs than other species, particularly in clade B, C and J (27, 12 and 16, respectively) (Fig. 1A). The numbers of predicted STs varied significantly among different species, which could be associated with their preferences for carbon sources and affect their growth on corresponding substrates (Cornell et al., 2007). For instance, *T. reesei* has a reduced number of the ST genes in most clades compared to other three studied species. Growth profiling of fungi on common mono- and disaccharides showed that *T. reesei* grew relatively poorly on D-galactose, L-rhamnose, D-galacturonic acid and D-glucuronic acid compared to other three fungi (see supplementary materials). *T. reesei* also showed reduced growth on lactose, maltose and sucrose, which is consistent with a small set of transporters related to disaccharides (clade B) and short oligomeric sugars (clade J). In contrast, *P. subrubescens* with relatively large ST repository showed good growth on most tested carbon sources.

Based on their close clustering with known STs and clear separation from other STs on phylogenetic tree, additional subclades were defined to assign sugar specificities to several putative STs (Fig. 1B, see supplementary materials). As the subclades typically only contain STs for a single type of compound, the characterized STs in them probably define the specificity of these subclades. Based on Fig. 1B, two distinct subclades, A1 and A2, were assigned as myoinositol and D-fructose STs clades, respectively, while subclade B1 was assigned as maltose STs. Subclades C1 and C2 contain hexose transporters. Notably, functionally characterized STs within subclade C1 contain both high and medium affinity D-glucose STs, while subclade C2 comprises mainly of high affinity D-glucose ST. Subclades D1 and D3 contain uronic acid STs, while subclade D2 contains quinic acid STs. Two subclades were found in Clade E, with E1 containing mainly D-xylose STs, while E2 contains proteins that transport both D-xylose and D-glucose. Subclades G1 and G2 contain STs for L-arabinose and D-glucose, respectively. A small subclade H1 was assigned as L-arabitol and L-arabinose STs consisting of single copy from each of four studied species, including the characterized LatA of *A. niger* and Lat2 of *Ambrosiozyma monospora*. In addition, two oligo-saccharide subclades J1 and J2 were assigned. Subclade J1 contains mainly lactose and cellobiose STs, while J2 has mainly cellobiose and xylobiose transporters.

These functional prediction based on phylogenetic analysis provides a more clear picture of the diversity of the sugar transportome of these fungi but also demonstrate the strong need for additional characterized STs, as currently no specificity or affinity can be predicted for several subclades of the phylogenetic tree. This tree will guide the selection of the most relevant ST candidates to be characterized.

3.2. Diverse expression profile of sugar transporters

To investigate the transcriptomic response of the predicted STs to different sugars, a large RNA-seq data set of fungi grown on nine different monosaccharides were comparatively analyzed. Overall, gene expression of the STs across different clades and species displayed high diversity (Fig. 1B).

A considerable number of STs showed specific sugar inducing patterns on the transcriptome data, some of which are consistent with the specificity prediction based on phylogenetic analysis, which strongly indicates their probable functions. For instance, subclades D1 and D3 containing characterized uronic acid transporters, were most induced by D-galacturonic acid and D-glucuronic acid. In the D-xylose related subclade E2, one ortholog group contains the functionally characterized D-xylose STs Str1, XtrD, XltA and two putative *P. subrubescens* STs (Ps_8243, Ps_4397). All members of this group were higher expressed on D-xylose and L-arabinose than other sugars, supporting the ability to transport pentose for the two putative *P. subrubescens* STs. STs within subclade G1 and H1 containing characterized L-arabinose ST TrXlt1 (Tr 118788) and L-arabitol ST LatA (Ang_4757) showed higher expression on L-arabinose than other sugars. This result strongly supports the function of newly predicted L-arabinose STs (Ang_8663, And_8480 and Ps_13633) and L-arabitol and L-arabinose STs (And_579, Ps_10020 and Tr_112629). In addition, three STs of subclade A2 (Ang_11807, Ps_653, Ps_1399) and two STs of subclade J1 (And_8915, Ps_11414) showed higher expression on D-fructose and D-galactose, respectively, which is consistent with the predicted function based on ortholog inference. Three STs (Ang_4244, And_9765 and Ps_13339) from unknown ST clade I showed a strong L-rhamnose specific inducing pattern, suggesting that they may be L-rhamnose transporters. These ST candidates with specificity supported by both phylogenetic and transcriptome data could be prioritized for further biochemical characterization.

Due to the lack of corresponding sugar growth condition in this study, relative low expression were detected in several ST (sub-)clades on nine tested sugars, such as the putative myoinositol STs in subclade A1, quinic acid STs in subclade D2 and disaccharide and oligosaccharide transporters within clade B and clade J. Additionally, several characterized STs and their orthologs showed complex expression profiles. For example, in subclade C2, the characterized Stp1 of *T. reesei,* MstA and MstH of *A. niger* and HxtB of *A. nidulans* showed high expression in most of the tested conditions, while their close homologs within this clade showed much lower or selective expression on the tested sugars. The diverse expression profile among STs from the same clade (even ortholog group) could be linked with their complex transcriptional regulation (Peng et al., 2018). For instance, the expression of the *A. niger* D-xylose transporter gene *xltA* (Ang_11715) was significantly reduced in Δ*xlnR* (cellulose degradation regulator deletion mutant), while the expression of *xltB* (Ang_9716) was mainly reduced in Δ*rhaR* (rhamnose utilization regulator deletion mutant) (Peng et al., 2018). In *T. reesei*, the expression of many STs has been shown to be modulated by the transcriptional regulators Xyr1 (dos Santos Castro et al., 2016) and Ace3 (Häkkinen et al., 2014). However, the ortholog of the *T. reesei* Ace3 is not present in Eurotiomycetes (Benocci et al., 2017). In addition, it has been shown

that the same regulator can control different sets of target genes in different fungi (Klaubauf et al., 2014), which likely also applies to the sugar transporter encoding genes under their control.

3.3. Affinity of STs

Evolution of the ST genes in fungi is governed by the adaptation of the fungi to their habitats. STs with different levels of affinity, including low- (K_m > 50 mM), moderate- (K_m from 1 mM to 50 mM) and high-affinity (K_m < 1 mM), have been reported for many fungi (see supplementary materials). For example, *S. cerevisiae* that is highly specialized in hexose utilization, possesses a large set of hexose transporters (Wieczorke et al., 1999) including 16 Hxt STs, a D-galactose permease Gal2 and two sensors Rgt2 and Snf3 classified into hexose, pentose, polyol clade C (see supplementary materials). The different affinities of these transporters for D-glucose, such as Hxt6, Hxt7 and Gal2 with moderate affinity ($K_{m(D\text{-glucose})}$ = 1–2 mM), Hxt2 and Hxt4 with moderate affinity ($K_{m(D\text{-glucose})}$ approx. 10 mM) and Hxt1 and Hxt3 with low affinity ($K_{m(D\text{-glucose})}$ = 50–100 mM), allow *S. cerevisiae* to respond to diverse sugar concentrations. The generalist species *A. niger* has three functionally characterized high affinity D-glucose STs in clade C (Fig. 1B), which matches its natural biotope that would typically have low free sugar concentrations.

D-xylose is the second most abundant monosaccharide in lignocellulose and therefore corresponding STs are interesting candidates e.g., for fungal cell factory applications. Several STs from clade E (Fig. 1B, see supplementary materials) with diverse kinetic properties have been characterized, including *A. niger* XltA and *T. reesei* Str1. *A. niger* XltA is a high affinity D-xylose transporter ($K_{m(D\text{-xylose})}$ = 0.09 ± 0.03 mM) with a similar affinity for D-glucose ($K_{m(D\text{-glucose})}$ = 0.07 ± 0.01 mM), while *T. reesei* Str1 that is closely clustered with XltA, shows even higher affinity for D-glucose ($K_{m(D\text{-glucose})}$ = 0.01 ± 0.00 mM) than for D-xylose ($K_{m(D\text{-xylose})}$ = 5.70 ± 0.19 mM). In contrast, an *A. niger* moderate affinity D-xylose transporter XltB ($K_{m(D\text{-xylose})}$ = 15.0 ± 4.50 mM) has been reported to be specific for D-xylose based on growth and competition experiments (Sloothaak et al., 2016b). *A. niger* XltC and *T. reesei* Str3 have moderate affinity for D-xylose ($K_{m(D\text{-xylose})}$ = 4.71 ± 1.04 mM and 2.19 ± 0.29, respectively) and transport a wide range of hexoses (Sloothaak et al., 2016b). These STs are present in clade C, E and G, respectively (Fig. 1B), which may explain their different substrate profiles. Also, D-glucose transporters from clade C share the transport capability with STs of clade E, i.e., *A. niger* high-affinity D-glucose transporter MstA ($K_{m(D\text{-glucose})}$ = 0.025 ± 0.01 mM) and *A. nidulans* moderate-affinity D-glucose transporter HxtB ($K_{m(D\text{-glucose})}$ = 15.54 ± 3.2 mM), which both have high affinity for D-xylose ($K_{m(D\text{-xylose})}$ = 0.30 ± 0.10 mM and 0.54 ± 0.08 mM, respectively) (see supplementary materials).

Although several fungal studies have focused on the utilization of D-xylose, the pentose sugar, L-arabinose, is also abundantly present e.g., in pectin rich industrial waste streams (Müller-Maatsch et al., 2016). Therefore, a set of L-arabinose transporters would expand the industrial utilization of a wider range of plant biomass derived sugars. This is exemplified by characterized L-arabinose transporters from clade G with various affinities (see supplementary materials), such as those from the ascomycete yeast *Scheffersomyces stipitis* (SsAraT, $K_{m(L\text{-arabinose})}$ = 3.8 ± 1.7 mM), and the filamentous fungi *Penicillium chrysogenum* (PcAraT, $K_{m(L\text{-arabinose})}$ = 0.13 ± 0.03 mM) and *T. reesei* (Tr_118788 / TrXlt1, $K_{m(L\text{-arabinose})}$ = 0.102 ± 0.02 mM). *T. reesei* ST Tr_118788, as originally identified and characterized as D-xylose transporter TrXlt1 (Saloheimo et al., 2007), is less inhibited by D-xylose and D-glucose than the other characterized L-arabinose transporters (Havukainen et al., 2021) stressing its high specificity for L-arabinose.

In this study, no strong association between affinity and expression of STs was identified. However, previous studies have suggested that high affinity STs prefer to be induced at low sugar concentration, while low affinity STs mainly function at high concentration (Lagunas, 1993). Given only a single moderate sugar concentration (25 mM) was tested in this study, its association between affinity and expression of STs could not be evaluated. Three high-affinity D-glucose STs of *A. niger*, MstA, MstH and MstG, showed relatively high expression on D-glucose compared to many of the other sugars (Fig. 1B). Similar expression patterns were observed for *A. niger* XltA and XltC on pentose. In addition, nine of 28 *A. niger* STs with extremely low expression level (with maximum FPKM < 10 in all tested conditions) showed moderate expression (FPKM > 50) in conditions with low level of free sugars, e.g., on aromatic components (Lubbers et al., 2019) and crude plant biomass (Peng et al., 2021) (data not shown). These lowly expressed STs have often been ignored in previous studies, but they could function as high affinity STs that are only activated in extremely low sugar conditions.

3.4. Co-expression analysis of STs and other PBC genes

Co-expression analysis has been commonly used to identify genes that are involved in similar or closely related biological processes or pathways (Stuart et al., 2003), which was applied to infer biological processes for hypothetical genes in *A. niger* (Schäpe et al., 2019). Here, co-expression between STs and CAZymes, TFs and SCEs were analyzed, which are the crucial enzymes and proteins involved in fungal plant biomass conversion (Peng et al., 2021). As expected, many CAZymes and SCEs were tightly co-expressed with STs, whereas only few TFs, e.g., RhaR, GaaR, ClrA/B, Xyr1 and Ace3 were identified to be co-expressed with STs, likely due to the fact that many of the TFs are under post-transcriptional rather than transcriptional control themselves (Fig. 2).

Several STs showed conserved co-expression patterns across multiple species. For instance, expression of *gatA* (Ang_958), encoding a D-galacturonic acid transporter in *A. niger*, correlated with 17 CAZyme, 20 SCEs and 11 other predicted ST encoding genes (see supplementary materials). Among the co-expressed genes, 13 genes encode pectinolytic enzymes, while three encode D-galacturonic acid catabolic and two L-rhamnose catabolic enzymes, and four encode STs from clade D with uronic, and quinic acid STs. This is in line with previous studies that fungal pectin utilization is tightly controlled at the expression level (Peng and de Vries, 2021). Similar co-expression patterns were also identified for the D-galacturonic acid ST encoding *gatA* ortholog in *P. subrubescens* (Ps_11846), but less PBC related genes were co-expressed with ortholog of *gatA* in *A. nidulans* (And_3524) and characterized *gat1* in *T. reesei* (Tr_109101) (see supplementary materials), revealing different regulatory organization in these species. In addition, *xltA* (Ang_11715), encoding a D-xylose ST of *A. niger*, was co-expressed with 13 genes encoding xylan or xyloglucan degrading CAZymes, one encoding a pentose catabolic enzyme, two enzymes related to the pentose phosphate pathway and *xltC* (Ang_10052), encoding another D-xylose ST. Most of these correlated PBC genes were highly expressed on D-xylose and L-arabinose (see supplementary materials). A similar co-expression profile was observed for its ortholog (Ps_8243) in *P. subrubescens*.

The co-expression analysis not only validated the function of previously characterized STs or their homologs, but also indicated possible functions for newly predicted STs. For example, the phylogenetic analysis assigned *A. niger* Ang_2828 and *A. nidulans* (And_8485) and *P. subrubescens* (Ps_4229) to clade H consisting of glycerol, arabitol and pentose STs, while the co-expression profile suggested them to be L-rhamnose STs (see supplementary materials). For example, Ang_2828 was highly correlated with a characterized TF (RhaR, Ang_1496) regulating L-rhamnose utilization (Gruben et al., 2014), 13 CAZyme encoding genes related to the degradation of pectin (with L-rhamnose as one of major components), as well as the gene encoding the characterized L-rhamnose ST (RhtA, Ang_3278, not a member of PF00083) (Sloothaak et al., 2016a). In previous study, expression of Ang_2828 was significantly down-regulated in the *A. niger* mutant strain Δ*rhaR* (Peng et al., 2018). Moreover, *A. niger* Δ*rhtA* mutant showed reduced but not completely abolished growth on L-rhamnose (Sloothaak et al., 2016a),

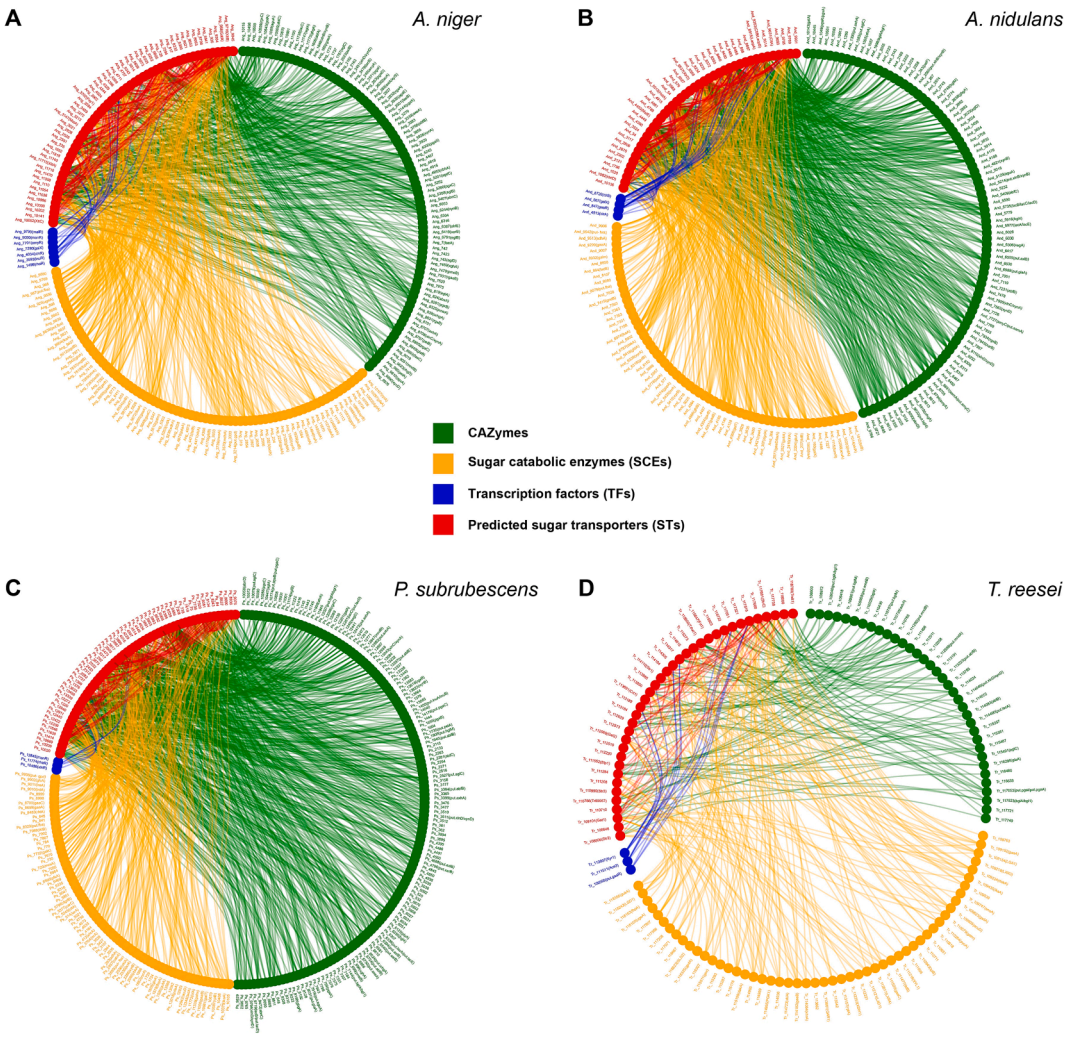

Fig. 2. Co-expression between STs and other plant biomass conversion (PBC) genes. The correlation networks between STs and CAZymes, sugar catabolic enzymes (SCEs) and transcription factors (TFs) were plotted for each species: (A) *A. niger*, (B) *A. nidulans*, (C) *P. subrubescens* and (D) *T. reesei*. Gene names are given in color corresponding their functional groups.

which supports the presence of other STs involved in uptake of L-rhamnose. These ST candidates with inconsistent function prediction from co-expression and phylogenetic analysis require further experimental confirmation.

4. Conclusion

In this study, the ST genome content of four ascomycete fungi and their transcriptome profiles were compared. This provides new insights into the diversity of STs across fungi. The predicted functions of STs will also facilitate their biochemical characterization. Future studies should cover broader experimental conditions, and characterize more STs from less-studied clades, such as clade B, H and J. The further integration of additional omics data, structural and biochemical information could help to reveal the full picture of the diversity of fungal sugar transportomes and enable the selection of the best transporters for specific biotechnological applications.

CRediT authorship contribution statement

Li Xu: Formal analysis, Visualization, Writing – original draft. **Jiajia Li:** Formal analysis. **Victor M. Gonzalez Ramos:** Formal analysis. **Christina Lyra:** Formal analysis. **Ad Wiebenga:** Formal analysis. **Igor V. Grigoriev:** Writing – review & editing. **Ronald P. de Vries:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Miia R. Mäkelä:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Mao Peng:** Conceptualization, Formal analysis, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The reads from each of the transcriptome sequencing (RNA-seq) samples were deposited in the Sequence Read Archive at NCBI under the accession numbers: *A. niger* SRP448993, SRP449003–SRP449007, SRP449023, SRP449039, SRP449049, SRP449062, SRP449079–SRP449081, SRP449083–SRP449085, SRP449089, SRP449068–SRP449070, SRP449098, SRP449125, SRP449138, SRP449141, SRP449142, SRP449151, and SRP449193; *A. nidulans* SRP262827- SRP262853; *P. subrubescens* SRP246823-SRP246849; *T. reesei* SRP378720-SRP378745.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.130006.

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