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A novel major facilitator transporter TrSTR1 is essential for pentose utilization and involved in xylanase induction in *Trichoderma reesei*

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ABSTRACT

Trichoderma reesei (teleomorph *Hypocrea jecorina*) is an industrially important filamentous fungus for glycoside hydrolases production, with its xylanolytic enzymes widely applied in many areas. However, the molecular mechanisms underlying xylanase expression are still insufficiently understood. In particular, the effect of sugar transporter on the induction of xylanase expression is unclear. In this work, we identified a novel major facilitator transporter TrSTR1 that is capable of transporting xylose by using a xylose utilization system in *Saccharomyces cerevisiae*. In *T. reesei*, TrSTR1 is essential for the utilization of D-xylose, L-arabinose, and even their downstream metabolites D-xylitol and L-arabitol. TrSTR1 is also involved in the induction of xylanase expression since both the xylanase activity and extracellular protein concentration in the Tu6△*str1* strain were decreased, which further confirmed by a qRT-PCR analysis of the transcript levels of the key transcriptional regulators. Our observations provide new insights into connections between pentose utilization and xylanase production in *T. reesei*.

1. Introduction

The sordariomycete *Trichoderma reesei* (teleomorph *Hypocrea jecorina* [1]) is widely used in industrial applications, including the paper, pulp [2], textile [3], food, and feed industries [4] because of its high secretory capacity of glycoside hydrolases. In recent years, the expression and regulation of glycoside hydrolases have been intensively studied in *T. reesei*. Several key transcriptional regulating factors (XYR1, ACE1, ACE2, CRE1, and Hap2/3/5 complex) [5] that are directly related to cellulases and/or hemicellulases induction have thus been identified. However, the molecular mechanism by which *T. reesei* senses extracellular nutrients and transmits signal for glycoside hydrolase gene expression remains insufficiently understood. Most cellulolytic and hemicellulolytic enzymes are adaptive enzymes, meaning that the expression of these enzymes is induced in the presence of certain carbon sources (e.g. cellulose and hemicellulose), whereas the induction will be antagonized in the presence of simple sugars such as glucose and fructose [6]. To trigger the production of lignocellulolytic enzymes, the insoluble polysaccharides must be degraded to soluble oligosaccharides, which then can be absorbed by the fungal cells, acting either as principal sources of carbon and energy or as direct or indirect inducer (i.e. the precursors of the true inducers) molecules [7,8]. In either case, the system of sugar uptake and utilization is critical for gene induction or repression.

To assimilate soluble oligo- and monosaccharides, *T. reesei* has developed a variety of transporters. Saloheimo et al. expressed a *T. reesei Trxlt1* gene encoding a putative xylose transporter in a modified recombinant *Saccharomyces cerevisiae* strain and found that it could enhance its xylose uptake capacity [9]. Moreover, a lactose permease, Trire2:3045, which is essential for lactose uptake and utilization, has been identified using system analysis of the *T. reesei* transcriptome [10]. The follow-up experiments suggested that Trire2:3045 (i.e., Crt1) also plays a pivotal role in cellulase expression [10,11]. Moreover, a sugar transporter, Stp1, capable of

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transporting cellobiose and glucose has been identified. Deleting Stp1 enhances cellulase induction [11].

Regarding the utilization of heterogeneous xylan by *T. reesei*, the production of xylanases is regulated by different inducers [12]. However, the influence of the utilization of specific sugars on the relevant xylanase gene expression is largely unknown. In the present work, we identified a novel gene *Trstr1* that encodes a putative sugar transporter. We provide evidence that *Trstr1* capable of transporting D-xylose is essential for both pentose (i.e. D-xylose and L-arabinose) and pentanol (i.e. D-xylitol and L-arabitol) utilization by *T. reesei*. A TrSTR1-deletion strain was impaired in xylanase production. Our results shed new light on the connections between pentose utilization and xylanase production.

2. Material and methods

2.1. Microbial strains and growth conditions

The *Escherichia coli* DH5α strain was used as a host strain for cloning purposes. Oligonucleotides used in this study are listed in Table S1 in the supplemental materials.

The *S. cerevisiae* strain EBY.VW4000 [13] is a hexose-null mutant and was used for the initial functional analysis of TrSTR1. The relevant mutants derived from EBY.VW4000 (The construction of these mutants is shown in supplemental material) were grown in minimal medium (0.67% yeast nitrogen base without amino acids plus required amino acids) containing 2% maltose at 28 °C. Uracil or tryptophan was lacking for transformants selection.

The *T. reesei* Tu6△*tku70* strain [14], which is a uridine auxotrophic strain derived from the TU-6 (ATCC MYA-256) strain and has the *tku70* gene knocked-out, was used as a parental strain to construct the *T. reesei* mutants. The mutants Tu6::*pyr4* (the strain transformed with the *pyr4* cassette), Tu6△*str1* (△*str1::pyr4*$^+$), Tu6Re*str1* (Tu6△*str1::str1, pyr4*), and Tu6*str1*$^+$(PcDNA1-*str1::pyr4*$^+$) (The construction of these mutants is shown in supplemental material) were used throughout this study. The *T. reesei* strains were grown for 4–6 days in the minimal medium [15] containing 1% (w/v) of a selected sugar as the sole carbon source or the potato dextrose agar (PDA) plates at 30 °C. When necessary, 5 mM of uridine was added. For liquid cultures, strains were precultured in 1-l Erlenmeyer flasks on a rotary shaker (220 rpm) at 30 °C for 24 h in 250 ml of MM medium with 1% (w/v) glycerol as the carbon source. Pre-grown mycelia were harvested and washed with sterile water, and then, equal amounts were resuspended in the MM medium containing 1% of the corresponding carbon source. Incubation was continued for 12 h, 24 h, and 48 h when cultivated on D-xylose- or L-arabinose-containing media.

For analysis of the effect of the *Trstr1* deletion on different carbon sources, equal amounts of fresh spores (10^5 spores) were inoculated on the MM plates containing 1% of the corresponding carbon source. The colony diameters were measured every 12 h.

2.2. Determining the subcellular localization of TrSTR1 in S. cerevisiae

Subcellular localization of the STR1-GFP fusion in transformed yeast cells was determined under a fluorescence microscope (Zeiss Axiovert 200, Zeiss, Thornwood, NY).

2.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

For RNA extraction, mycelia were harvested at the indicated time points and then stored in liquid nitrogen. The total RNA was isolated with TRIzol reagent (Life Technologies, Gaithersburg, MD). cDNA was synthesized from the total RNA using the Superscript III First-strand Synthesis kit (Invitrogen, Carlsbad, CA). Real-time PCR was then carried out using the cDNAs as templates in PIKO REAL 96 Real-time PCR system (Thermo Fisher Scientific, Waltham, MA) using TransStart™ Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China). All PCRs were performed in triplicate in 10 μl containing 5 μl 2 × TransStart™ Top Green qPCR SuperMix, 0.2 μM forward primer, 0.2 μM reverse primer, and 0.5 μl 20-fold diluted cDNA template. The PCR steps were: 30 s initial denaturation at 95 °C, followed by 40 cycles of 5 s at 94 °C and 30 s at 60 °C. A melting curve analysis with a temperature gradient of 0.1 °C from 50 °C to 99 °C was performed. PiKoReal software 2.1 was used to calculate the CT value. The fold change in target gene cDNA relative to the actin control was determined by the ΔΔCT method [16]. The values given in the figures are means for three independent experiments. Error bars indicate standard deviations.

2.4. Enzyme assay and protein measurement

The xylanase activity was measured by the dinitrosalicylic acid (DNS) method [17] using birch-wood xylan (Sigma–Aldrich) as the substrate. One unit of activity was defined as the amount of enzyme required to release 1 μmol of xylose reducing equivalents per minute under the defined assay conditions. The protein concentration was determined using the Easy Protein Quantitative kit (TransGen Biotech, Beijing, China).

3. Results

3.1. Identification of a putative sugar transporter in T. reesei by bioinformatic analysis

By searching the *T. reesei* genome with the BLAST-X algorithm using selected reported mono- and disaccharide transporter cDNA sequences from several fungi in the NCBI GenBank as enquiries, we identified five amino acid sequences (GenBank accession no. EGR46289, EGR46373, EGR49690, EGR51026, and EGR44682) that belong to the major facilitator superfamily. They were designated TrSTR1, TrSTR2, TrSTR3, TrSTR4, and TrSTR5, respectively. Four of these putative transporters, TrSTR2, TrSTR3, TrSTR4, and TrSTR5, are highly similar to the reported disaccharide transporters in their amino acid sequences and the remaining transporter, TrSTR1, shows highly homologous with monosaccharide transporters. Here, we focused on the TrSTR1 protein because the *Trstr1* gene (Trire2:50894) was apparently up-regulated with different carbon sources in comparison with glucose [10,18,19]. The phylogenetic tree (Fig. 1) indicates that TrSTR1 is segregated apart from the disaccharide transporters of *S. cerevisiae, Neurospora crassa, Emericella nidulans*, other related fungi, and the plant *Arabidopsis thaliana*. However, TrSTR1 is more closely related to the hexose transporters of *E. nidulans, Kluyveromyces. lactis*, and *Trichoderma. harzianum.* The characterized hexose transporter TrHXT1 [20] and xylose transporter TrXLT1 [9] in *T. reesei* are also in this cluster, suggesting that TrSTR1 is a putative monosaccharide transporter.

The annotated open reading frame (ORF) of *Trstr1* is 1704 bp long, interrupted by seven introns, and predicted to encode a membrane protein containing 567 amino acid residues. The protein with the highest identity (95%) to TrSTR1 in the non-redundant protein database in NCBI was a hypothetical protein, from *Trichoderma virens* Gv29-8. As predicted by TMHMM version 2.0 [21], TrSTR1 is a MFS transporter with an 11-transmembrane domain (TMD) topology and an extracellular termini. TrSTR1 has a large extracellular loop (between TMD1 and TMD2), a large intracellular loop (between TMD10 and TMD11), and a relatively long central loop that is shared by many reported sugar transporters. TrSTR1 also has the same five-element fingerprint signature as most sugar

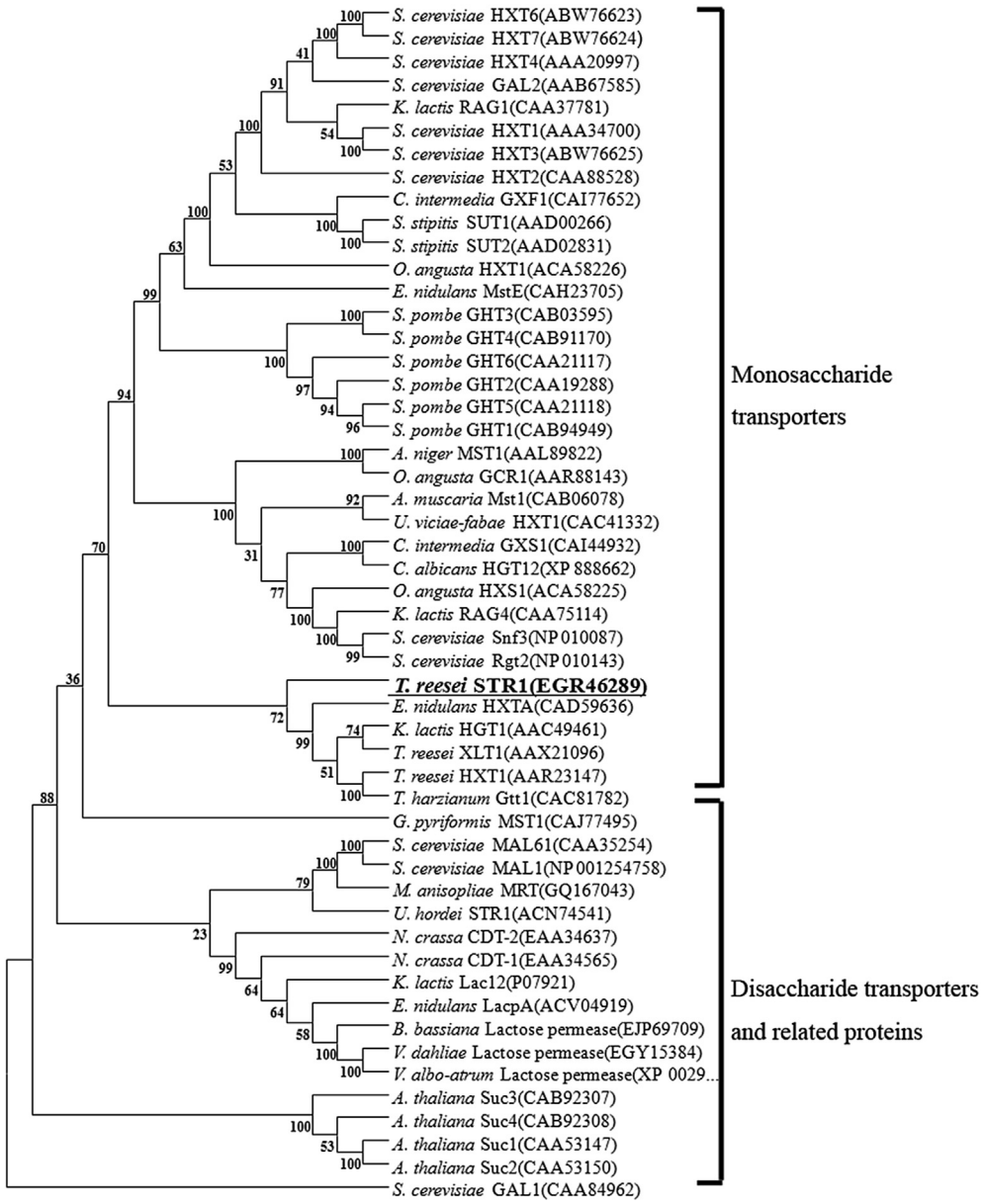

Fig. 1. Phylogenetic analysis of TrSTR1 and its homologous proteins. This analysis was carried out by MEGA4 software using the neighbor-joining method. Bootstrap values are adjacent to each internal node and represent the percentage of 1000 bootstrap replicates.

transporters [22] (Fig. 2A). The key residues that have been shown to be involved in either sugar specificity or substrate binding in some structure-solved sugar transporters were conserved in TrSTR1 [23–25] (Fig. 2A).

3.2. Phenotypic analysis of TrSTR1 in a hexose-null yeast *S. cerevisiae EBYVW.4000*

To characterize the function of a transporter in *T.reesei* or in some other fungi, a hexose-null yeast strain EBY.VW4000, which lacks the hexose transport activity due to the deletion of 20 hexose transporter genes [13], is often used for investigating its cellular location and substrate specificity [11,26]. In this study, a plasmid bearing a TrSTR1-GFP fusion construct was generated and transformed into the strain EBY.VW4000. Fluorescence can be observed predominantly on the cell surface (Fig. 2B), suggesting that TrSTR1 is likely localized to the yeast plasma membrane. In addition, a TrSTR1-expressing recombinant EBY.VW4000 strain was also prepared and analyzed for its capacity to utilize different carbon sources. The transgenic yeast strain cannot grow on pentose, such as D-xylose and L-arabinose, due to the lack of pentose metabolism in *S. cerevisiae*. Interestingly, the strain was also unable to grow on several hexoses, such as D-glucose, D-fructose, D-galactose, and D-mannose (data not shown). To further explore the function of TrSTR1, the *S. cerevisiae* strain YXL4000 derived from *S. cerevisiae* EBY.VW4000 containing the expression cassettes needed for xylose metabolism and TrSTR1 was constructed and analyzed for the capacity to utilize D-xylose, the *S. cerevisiae* strain YXL4000 was inoculated into maltose medium, followed by cultures in shake flasks on liquid media containing 20 g/L D-xylose. No growth of the strain YXL4000 was observed after 2 days, however, growth was obtained after a long lag of 15 days, whereas the strain containing only the empty vector didn't grow even after 1-month culture on D-xylose (Fig. 2C). It should be noted that a similar phenomenon has

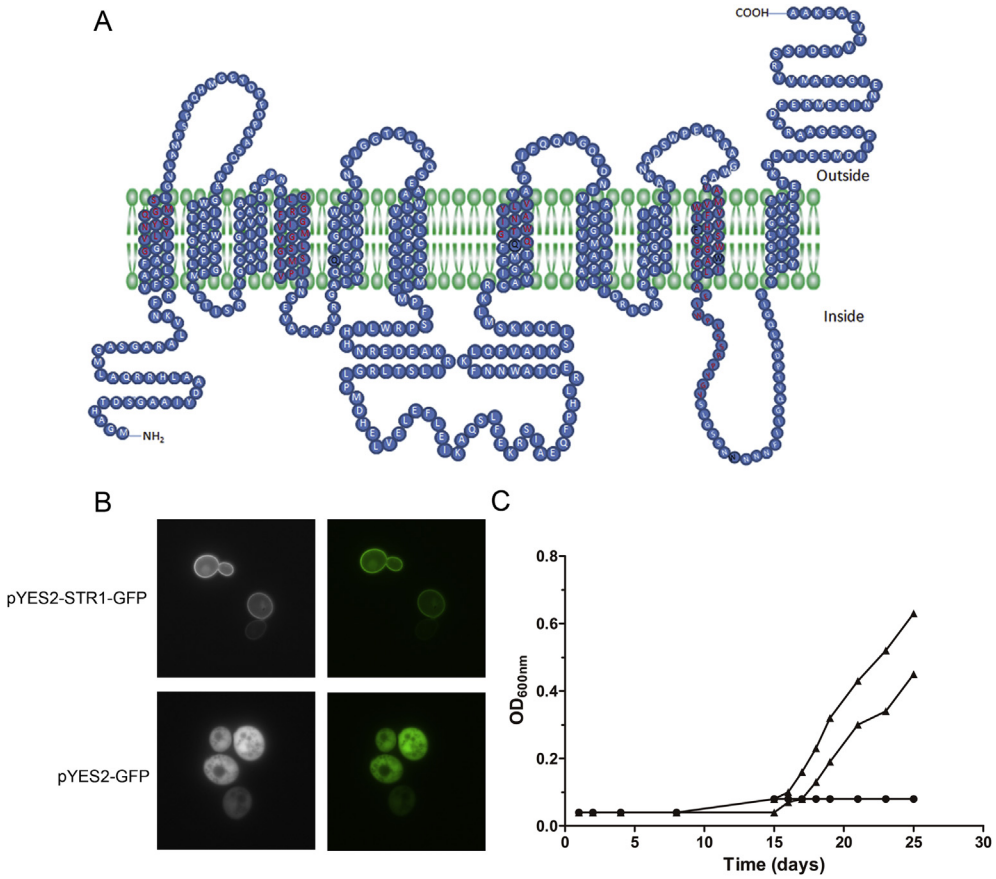

Fig. 2. Functional analysis of TrSTR1 in *S. cerevisiae*. (A) Schematic representation of the predicted topology of TrSTR1 by the program TMHMM version 2.0. The fingerprint sequences of the sugar transporter family are in red font. The conserved amino acids that have been shown to function as sugar transporters/sensors are in black font. (B) Subcellular localization of TrSTR1 in a recombinant *S. cerevisiae* strain. The yeast EBYVW.4000 transformed with an STR1-GFP-encoding plasmid pYES2-STR1-GFP (top panels) or a sole GFP-encoding plasmid pYES2-GFP (bottom panels) were monitored by fluorescence microscopy. Right panel: fluorescence; left panel: bright field. (C) Growth of the *S. cerevisiae* EBYVW.4000 harboring the xylose-utilization pathway and also transformed with *Trstr1* (two independent transformants are shown, ▲) or empty vector (●) on D-xylose. Strains are inoculated in liquid medium with D-xylose as sole carbon source. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previously been observed for *Trxlt1* selection in a *S. cerevisiae* strain [9]. Analysis of the culture medium showed the D-xylose consumption was consistent with the growth. To address whether the growth on D-xylose was caused by the mutations of *Trstr1* gene that had occurred in yeast itself, the *Trstr1* gene was amplified using the plasmid isolated from the strain YXL4000 as the template and sequenced. No mutations were observed compared to the original *Trstr1* gene. Our results suggested the capability of TrSTR1 to transport D-xylose.

3.3. Deletion of Trstr1 impaired the growth of T. reesei

The yeast experiment demonstrated that TrSTR1 is able to transport D-xylose. To verify its function in *T. reesei*, we further deleted *Trstr1* in the genome of *T. reesei* Tu6△*tku70* strain [14], generating a Tu6△*str1* strain. To examine the effect of *Trstr1* deletion on different carbon sources, both the parent strain Tu6 and the deletion strain Tu6△*str1* were cultured on minimal medium (MM) plates supplemented with an array of sugars and sugar alcohols, including D-glucose, D-fructose, D-galactose, D-xylose, L-arabinose, D-mannose, sucrose, D-(+)-cellobiose, trehalose, lactose, maltose, D-(+)-raffinose, D-xylitol, L-arabitol, D-sorbitol, and glycerol, as the sole carbon source for six days. While the parent strain Tu6 was able to form normal colonies, the deletion strain Tu6△*str1* showed a delay of approximately 48 h in its growth, leading to the decrease of its hyphal density on most agar plates. After 48 h, Tu6△*str1* strain had comparable hyphal extension rates to the parent strain Tu6 on the carbon sources except pentose and pentanol (e.g., D-xylose, L-arabinose, D-xylitol, and L-arabitol) (Fig. 3). On the MM-plates with pentose and pentanol as the sole carbon source, the mutant strain exhibited severe growth defects. Moreover, it is worth noting that the colonial morphology of Tu6△*str1*strain changed on some carbon sources (e.g., D-mannose, D-(+)-cellobiose, and trehalose). To further verify this phenotype in *T. reesei*, another three mutant strains (i.e., a control strain Tu6::*pyr4*, a *Trstr1* complement strain Tu6R*str1* and a *Trstr1* overexpressing strain Tu6*str1*$^{+}$) were constructed. These mutants were cultured on D-xylose, L-arabinose, D-xylitol, and L-arabitol (Fig. S1). All of these mutants, except the *Trstr1*-deletion strain, were able to utilize the carbon sources investigated, indicating that the growth deficiency of *T. reesei* on pentose and pentanol is indeed caused by *Trstr1* deletion. These results suggest that *Trstr1* is involved in the utilization of a variety of carbon sources in *T. reesei* and more importantly, essential for pentose and pentanol utilization.

3.4. Expression of xylanolytic enzymes was affected by Trstr1 deletion

The expression of the xylanolytic enzyme-encoding genes is induced by hemicelluloses, such as xylan, or their respective

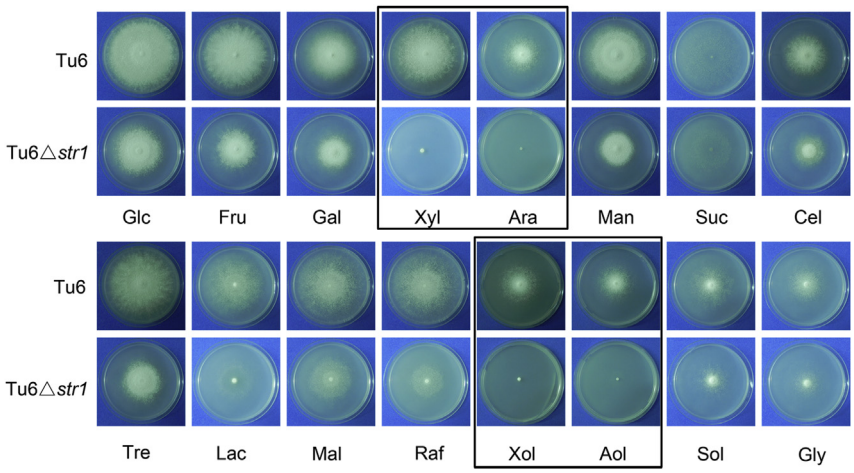

Fig. 3. Growth of *T. reesei* Tu6 and Tu6△ *str1* strain on different carbon sources. Pictures were taken at 144 h after incubation of the two strains on plates of MM supplemented with glucose (Glc), D-fructose (Fru), D-galactose (Gal), D-xylose (Xyl), L-arabinose (Ara), D-mannose (Man), sucrose (Suc), D-(+)-cellobiose (Cel), trehalose (Tre), lactose (Lac), maltose (Mal), D-(+)-raffinose (Raf), D-xylitol (Xol), L-arabitol (Aol), D-sorbitol (Sol), or glycerol (Gly).

hydrolysis products, such as xylose [6] and arabinose [12], in a concentration-dependent manner [27,28]. Because D-xylose and L-arabinose utilization was negatively influenced by *Trstr1* deletion, it seemed very likely that the expression of xylanolytic enzymes would also be affected. The mutant strains, including Tu6::*pyr4*, Tu6△*str1*, Tu6R*estr1*, were all pre-cultured in MM-glycerol for 24 h. Then, equal amounts of mycelia were transferred to MM containing xylan as the sole carbon source. The xylanase activity and secreted protein concentration in the supernatant samples were measured at different time intervals. Both the xylanase activity and secreted protein concentration were strikingly reduced in the deletion strain compared with Tu6::*pyr4* strain (Fig. 4A and B), indicating that TrSTR1 is involved in the expression of xylanases in *T. reesei.*

It is believed that the expression of the xylanolytic enzymes is regulated by several key transcription factors [5], such as XYR1, CRE1, ACE1, and ACE2. We consequently investigated if the deletion of *Trstr1* also influences the expression of these transcription factors on D-xylose [28]. The transcript level of the major transcriptional regulator *xyr1* in the Tu6△*str1* strain was significantly decreased compared with the Tu6::*pyr4* strain (Fig. 4C). Additionally, we found that the transcript abundance of *cre1* in the Tu6△*str1* strain is much higher (6.5-fold at 48 h) compared with that of its parent strain (Fig. 4D), and *Trstr1* had a negative effect on the transcription level of *ace1* and *ace2* (Fig. 4E and F). Our results show that *Trstr1* indeed affected the transcript abundance of the xylanase expression-related transcription factors, which may further lead to the alteration of the transcript patterns of xylanase genes.

4. Discussion

In nature, in the absence of simple sugars as carbon sources, *T. reesei* secrets extracellular lignocellulolytic enzymes to degrade heterogeneous plant cell wall polysaccharides. Subsequently, the degraded simple sugars can be assimilated for cell metabolism. In this process, transporters are crucial for *T. reesei* to sense and utilize sugars. Thus, studying the function of transporters will provide new insights for understanding the sensing mechanism in *T. reesei*. In this study, we identified a putative sugar transporter TrSTR1 that is essential for D-xylose, L-arabinose, D-xylitol, and L-arabitol utilization in *T. reesei*. To the best of our knowledge, this is the first report of a sugar transporter that is critical for both pentose and pentanol utilization in *T. reesei*.

The direct uptake of pentose via TrSTR1 in *T. reesei* was unable to be demonstrated presently because of the existence of other pentose transporters, e.g., TrXLT1, in *T. reesei*. However, the yeast experiment suggests TrSTR1 has the capacity of transporting D-xylose. The observed growth delay of the yeast strain on D-xylose might be due to the low efficiency of pentose metabolism derived from *P. stipitis*. Besides the capability of transporting D-xylose, our data also showed that TrSTR1 likely participated in the assimilation of L-arabinose, even D-xylitol and L-arabitol, in *T.reesei*, as Tu6△*str1* strain displayed severe growth defects on these carbon sources. The reason for *Trstr1*-mediated transport of both pentose and pentanol may be based on their structural similarity.

In this study, we further demonstrated that TrSTR1 is involved in xylanase induction. XYR1 has been proved as a central transcriptional regulator that controls hydrolytic enzyme system [29]. In addition, CRE1 is necessary for the full induction of *xyr1* on D-xylose. In this case, TrSTR1 may regulate xylanase gene expression mainly through affecting the expression of *xyr1* and *cre1*. Besides the effects on XYR1 and CRE1, we also found that TrSTR1 has a negative effect on ACE1 as well as ACE2. In this study, how *Trstr1* influences these transcriptional factors are unclear, however, it may directly result from the alteration of extracellular D-xylose concentration in Tu6△*str1* strain. Another hypothesis regarding the alteration of the expression levels of transcriptional factor genes is that TrSTR1 somehow affects the transmitting of the inducing signal. In any case, it is convincible that TrSTR1 plays an important role in xylanase gene expression, potentially through regulation of the transcript abundance of transcriptional factor gene.

In conclusion, our results show that *Trstr1* capbable of transporting D-xylose is essential for D-xylose and L-arabinose utilization in the industrially important cellulolytic filamentous fungus *T. reesei*. Moreover, *Trstr1* also plays important roles in regulating the expression of the glycoside hydrolases, such as the major xylanases. How *Trstr1* is regulated in *T. reesei* remains to be shown. This regulation may include the identification of key molecules in the signal transduction pathway that regulates *Trstr1* expression. Establishing the relationship between sugar metabolism and hydrolase expression will be a major challenge for future studies. Summarily, understanding the regulation of *Trstr1* itself would reveal important mechanisms regarding how *T. reesei* senses the extracellular environment to regulate glycoside hydrolase expression and would provide useful information for rationally designing genetically improved strains.

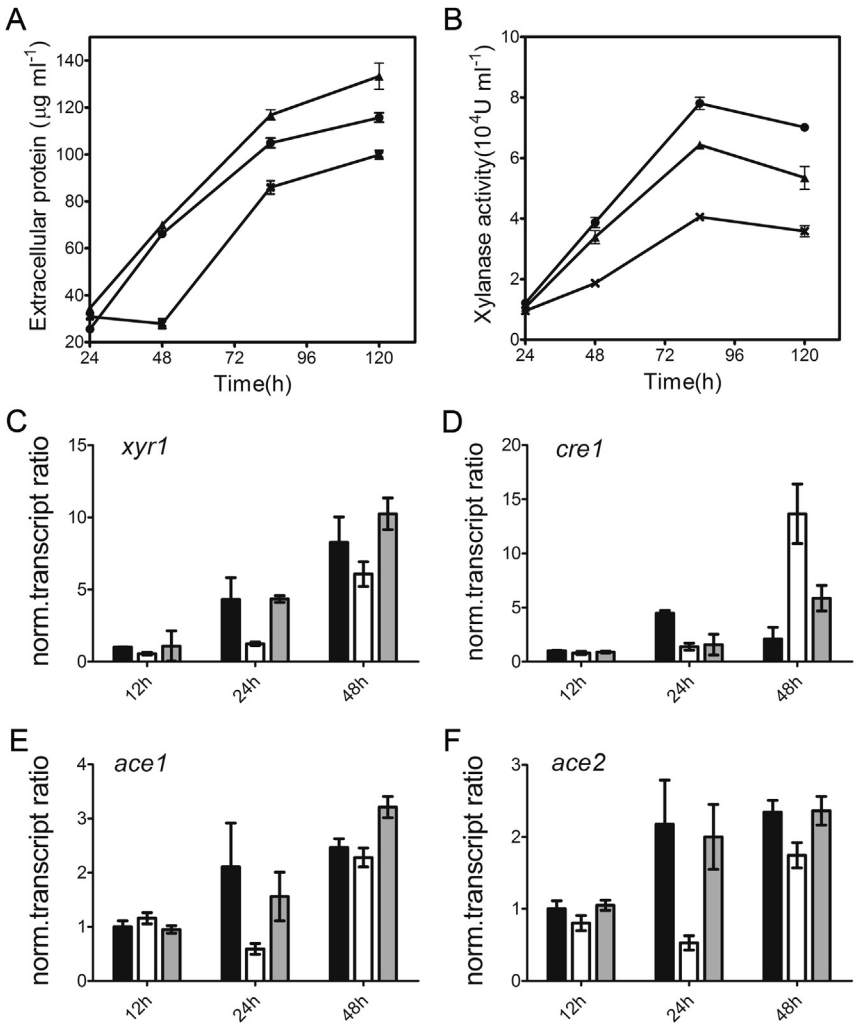

Fig. 4. Effects of deleting *Trstr1* on xylanase production and transcriptional analysis of *xyr1* (C), *cre1* (D), *ace1* (E), and *ace2* (F) in *T. reesei*. (A–B) Extracellular protein concentrations (A) and xylanase activity (B) in culture supernatants of Tu6::*pyr4* (●), Tu6△*str1* (×), and Tu6R*estr1* (▲). Strains were pre-cultivated in MM-glycerol for 24 h, and the mycelia were transferred to MM supplemented with 1% xylan as the sole carbon source. (C–F) The *T. reesei* strains Tu6::*pyr4* (black), Tu6△ *str1* (white), and Tu6R*estr1* (grey) were pre-cultivated in MM-glycerol for 24 h, and then, the mycelia were transferred to MM supplemented with D-xylose for 12 h, 24 h, and 48 h. The transcript level in Tu6::*pyr4* strain at 12 h was set as 1.0. The values for other strains were normalized by dividing the transcript abundance in those strains by that in strain Tu6::*pyr4*. Error bars indicate the standard deviation of three independent experiments.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.087.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.087.

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