Research Article
RNA Sequencing Reveals Xyr1 as a Transcription Factor Regulating Gene Expression beyond Carbohydrate Metabolism

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Received 31 August 2016; Accepted 6 November 2016

Academic Editor: Muhammad I. Rajoka

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Xyr1 has been demonstrated to be the main transcription activator of (hemi)cellulases in the well-known cellulase producer Trichoderma reesei [9]. This study comprehensively investigates the genes regulated by Xyr1 through RNA sequencing to produce the transcription profiles of T. reesei Rut-C30 and its xyr1 deletion mutant (Δxyr1), cultured on lignocellulose or glucose. Δxyr1 deletion resulted in 467 differentially expressed genes on inducing medium. Almost all functional genes involved in (hemi)cellulose degradation and many transporters belonging to the sugar porter family in the major facilitator superfamily (MFS) were downregulated in Δxyr1. By contrast, all differentially expressed protease, lipase, chitinase, some ATP-binding cassette transporters, and heat shock protein-encoding genes were upregulated in Δxyr1. When cultured on glucose, a total of 281 genes were expressed differentially in Δxyr1, most of which were involved in energy, solute transport, lipid, amino acid, and monosaccharide as well as secondary metabolism. Electrophoretic mobility shift assays confirmed that the intracellular β-glucosidase bgl2, the putative nonenzymatic cellulose-attacking gene cip1, the MFS lactose transporter lp, the nmrA-like gene, endo T, the acid protease pepA, and the small heat shock protein hsp23 were probable Xyr1-targets. These results might help elucidate the regulation system for synthesis and secretion of (hemi)cellulases in T. reesei Rut-C30.

1. Introduction

Lignocelluloses have long been recognized as the most abundant sustainable resources for the production of biofuels and other biomaterials [1, 2]. Cellulose must be hydrolyzed by cellulases to soluble carbohydrates to facilitate fermentation. The typical cellulase system consists of endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidase (BGL; EC 3.2.1.21), which act synergistically to hydrolyze cellulose to glucose [3]. The ascomycete Trichoderma reesei (anamorph of Hypocrea jecorina) has been used widely as a cellulase source since its discovery during World War II [4]. However, the cost of lignocellulolytic enzyme preparation is still among the major limitations in the development of an acceptable technology to convert lignocellulose to biofuels and other chemicals [5, 6]. Therefore, T. reesei should be further genetically engineered to acquire an improved strain for cellulase production.

Several transcription factors (TFs) involved in the regulation of cellulase gene expression have been identified in T. reesei, including the activators Ace2, Xyr1, and Hap2/3/5, as well as the repressor Ace1 and the carbon catabolite repressor Crel [7]. Xyr1, a homolog to XlnR in Aspergillus niger, is a zinc binuclear cluster protein binding to a 5ʹ-GGCTAA-3ʹ motif arranged as an inverted repeat [8]. It was demonstrated to play an essential role in transcriptional regulation of cellulolytic and xylanolytic genes, such as xyn1, xyn2, bxl1, abf2, cbh1, cbh2, egl1, and bgll [9, 10]. In addition, Xyr1 was reported to receive the lactose induction signal and regulate lactose metabolism by directly activating xylose reductase 1 transcription and indirectly influencing transcription of β-galactosidase 1 (bgal) [11]. Unlike xyr1 in T. reesei, the deletion of xlnR (ortholog to xlnR in Aspergillus) in Fusarium oxysporum affects only xylanase activity [12].

Recently, the TF xylan degradation regulator 1 (XLR-1), an ortholog to XlnR/Xyr1 in A. niger and T. reesei, was identified in Neurospora crassa. Deletion of xlr-1 in N. crassa prevented growth on xylan and xylose, but its cellulolytic activity was only slightly affected, indicating a different role from xyr1 in T. reesei [13]. Besides, secretome analyses of wild type and...
the xlnR/xhr1/xyr1 deletion mutants of five fungi showed that T. reesei Xyr1 has a different regulatory pattern compared to its orthologs in other fungi [14]. The above findings, combined with the demonstration that Xyr1 in T. reesei could bind not only to the 5'-GGCTAA-3' motif, but also to the 5'-GGC(A/T)2-3' motif [15], suggest that Xyr1 behaves as a pleiotropic regulator in T. reesei.

Recently, transcription profiling of the T. reesei Qm 9414 and its Δxyr1 mutant grown on cellulose, sophorose, and glucose were performed and defined the role of the transcriptional factor Xyr1 during cellulose degradation [16]. T. reesei mutant Rut-C30 is a hyperproducer of cellulolytic enzymes with its genome has been released [3, 17, 18]. Rut-C30 was obtained through several rounds of random mutagenesis from wt Qm6a. The rearrangement of chromosomes carrying genes encoding cellulolytic enzymes [19] and the missing >100 kb of genomic DNA [20] including the truncation of carbon catabolite repressor crel [17] may contribute to its high protein secretory ability and cellulase production. Portnoy et al. reported challenging results indicating that the full transcription of xyr1 required Crel in T. reesei Qm9414 under induction conditions [21]. Due to the special genetic background of Rut-C30, we assumed that its Xyr1 harbored rather special regulatory mechanisms compared to T. reesei Qm9414.

Wheat (Triticum aestivum L.) bran, which contains lignocelluloses as a major component, is rich in hemicelluloses, cellulose, and lignin [22, 23]. Therefore, wheat bran behaves as an inducer for lignocellulolytic enzymes. In this study, RNA sequencing (RNA-seq) was performed to investigate the functions of Xyr1 through comparison between a wild-type strain Rut-C30 and an xyr1 disruptant, under lignocellulose and glucose conditions. Our results shed new light on the mechanism by which Xyr1 controls cellulose and hemicellulose utilization and determines the pleiotropic functions of Xyr1. These new findings could offer strategies for strain improvement of T. reesei Rut-C30.

2. Materials and Methods

2.1. Fungal Strains and Cultivation Conditions. T. reesei Rut-C30 (ATCC 56765) was purchased from ATCC and its xyr1 deletion mutant strain Δxyr1 was constructed as following. Plasmids were propagated in Escherichia coli DH5α. The vector backbone used in constructing the plasmids was binary vector pCambia1300 (CAMBIA, Canberra, Australia). The E. coli cultivations were performed overnight at 37°C in Luria-Bertani (LB) medium plus kanamycin (100 µg mL⁻¹) as selective agent.

The xyr1 deletion vector was constructed using the binary vector pCambia1300 as a recipient. Primers used are given in Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4841756. First, the plasmid pSilent-1 [24] was used as template to amplify the ORF and terminator of hygromycin B phosphotransferase gene hph (conferring hygromycin B resistance) with primers HygXho and OrhHyg. Second, the pyruvate kinase (pki, GenBank accession number L07060) promoter was amplified by primers Ppki and Bampki with T. reesei Rut-C30 genome as template. Overlap PCR was conducted to fuse the pki promoter to hph ORF, and the PCR product was digested with BamHI and XhoI and ligated into the BamHI and XhoI digested pCambia1300, resulting in pPH. Then the 1.3 kb fragment for 5' region of xyr1 was amplified with primers xyr1Xho and xyr1Xhr. After digestion by XhoI, it was inserted to the XhoI digested pPH to generate pPH5X. At last, the 3' region of xyr1 was amplified with primers xyrbam and xyrsal and digested by BamHI and SalI before inserting into the corresponding restriction enzymes digested product of pPH5X to yield the xyr1 knockout vector pPHX. The resultant pPHX was transformed into T. reesei by Agrobacterium-mediated transformation as described previously [25]. Transformants were then subjected to verification of homologous recombination event. Primers X5 and TtrpC were used to amplify a fragment of 2.2 kb in the xyr1 knockout strain, and similarly primers X3 and Ppki were used to amplify a fragment of 2.3 kb in the xyr1 knockout strain and the results were further verified by sequencing the PCR products, while random insertion could not yield any specific PCR products.

The xyr1 recombination strain was constructed as: the phleomycin resistance gene (ble) [26] was used as a selection marker. The binary vector pPB was constructed in a similar way except replacing the hph ORF with ble. The xyr1 gene was amplified with primers X3BamH and X5BamH, and the terminator of xyr1 was amplified with primers Txyr5Xho and Txyr3Xho (Table S1). The PCR products were digested by BamHI and XhoI, respectively, and then inserted into corresponding site of the pPB to yield pPBReX. The transformation into Δxyr1 strain was performed as described above and transformants were selected on PDA containing phleomycin (4 µg mL⁻¹) as selection agent. The homologous integration of pPBReX at the xyr1 locus of the Δxyr1 strain was verified by PCR. Primers B3homo and pki-phe were used to amplify a fragment of 2.6 kb in the xyr1 homologous integration retransformant, and similarly, primers X5' and Ppki (Table S1) were used to amplify a fragment of 5.7 kb and the results were further verified by sequencing the PCR products, while random insertion could not yield any specific PCR products.

All of the fungal strains were maintained on potato-dextrose agar (PDA). Then, 10⁷ conidia of both strains were inoculated in 50-mL shake flasks containing 10 mL of Sabouraud’s dextrose broth (SDB) at 28°C for two days, and then the pregrown mycelia were collected by filtration through miracloth and washed with 0.9% NaCl, thoroughly. Then, the mycelia of both strains were transferred into the cellulase-inducing medium described by Ma et al. [25] which contains 2% wheat bran, 3% microcrystalline cellulose (Avicel), and the cellulase-repressing medium containing 2% glucose in place of wheat bran and Avicel, respectively. The flasks were incubated on an orbital shaker at 200 rpm, 28°C. For RNA isolation, the samples of mycelium were collected after 15 hours of cultivation and then subjected to RNA isolation. For fungal growth and protein secretion analysis, 500 µL samples were collected after being induced for 1 day, 2 days, 3 days, and 7 days. After centrifugation, the culture supernatant was subjected to electrophoresis,
extracellular protein concentration assay, enzymatic assays, and the mycelia which were used to quantify the biomass.

2.2. RNA Isolation, Sequencing, and Data Analysis. Fungal mycelia of four samples were harvested by filtration and centrifugation, frozen, and ground under liquid nitrogen. Total RNA were isolated by TRIzol (Invitrogen) according to the instruction, and RNA extracts were monitored by electrophoresis and quantified using a spectrophotometer. Total RNA were provided to Chinese National Human Genome Center at Shanghai for sequencing. mRNA purified from total RNA using the MicroPoly(A) Purist kit (Ambion) was used for library preparation and latter sequencing using the Illumina Hiseq2000 platform. The quality control was performed with FASTX-Toolkit (Version 0.0.13), and using the Illumina Hiseq2000 platform. The quality control was used for library preparation and latter sequencing. Genes purified genomic sequence and genestructure information for the significance of differences for multiple tests [32]. The package Cufflinks v0.83 [29]. Differential expression values (FPKM) was estimated with the program Cuffdiff from the GenomeCenteratShanghaiforsequencing.

2.3. Fungal Growth and Protein Secretion Analysis. The culture filtrate was collected by centrifugation at 4 °C, 8,000g for 10 min. For SDS-PAGE analysis, 10 μl of culture supernatants of T. reesei Rut-C30, its xyr1 deletion strain and xyr1 complementation transformant were subjected to SDS-PAGE. The protein concentration of each culture supernatant was determined using Modified Bradford Protein Assay Kit (Sangon, Shanghai, China) according to the manufacturer’s guidelines. The absorbance at 595 nm was measured with a Varioskan Flash microplate reader. Xylanase activities of different samples were determined according to Turunen et al. [39] with modifications. The diluted supernatants (50 μl) were incubated with 50 μl of 1% beechwood xylan (Sigma) dissolved in acetate buffer (100 mM, pH 4.8) at 50°C for 10 min. Then 100 μl of DNS was added and incubated at 95°C for 10 min, and the absorbance at 540 nm was measured. One unit of activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars per minute.

2.4. Quantitative Real-Time PCR (qRT-PCR). The RNA samples obtained from T. reesei Rut-C30 and Δxyr1 strain on either cellulose or glucose were reverse transcribed into cDNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer’s protocol. All real-time PCR were performed on a Mastercycler (Eppendorf, Hamburg, Germany) with twin.tech real-time PCR plate 96 (Eppendorf) and Mastercycler real-time PCR Film (Eppendorf). For the reaction the SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) was used for 25 μl assays. Primers used were given in Additional File, Table S1. Three replicates were performed per experiment. The amplification protocol consisted of an initial denaturation step (30 sec at 95°C) followed by 40 cycles of denaturation (5 sec at 95°C), annealing (30 sec at 58°C), and elongation (15 sec at 72°C). The data analysis was done using Realplex software (Eppendorf, Germany). The gpdA gene measurement was performed for reference calculation. Two independent experiments were performed and the data are averages of the duplicate determinations. For every experiment, two biological replicates were carried out with three technical replicates each. Student’s two-tailed t-test was performed using Excel 2007 (Microsoft, WA).

2.5. In Silico Analysis of Xyr1 Binding Sites of the 5′-Upstream Region of Genes. Gene models in the T. reesei Rut-C30 genome were downloaded from the T. reesei genome database at the Joint Genome Institute website (http://genome.jgi-psf.org/TrireRUTC30_1/TrireRUTC30_1.home.html), 5′ upstream regions (1 kb) for each gene were extracted from the scaffolds. The occurrence of 5′-GGC(A/T)₃, 5′ motifs and 5′-GGC(A/T)₃-5′ motifs in the 5′-upstream regions was determined for both strands.
2.6. Overexpression and Purification of the DNA Binding Domain of Xyr1. The DNA binding domain (residues 55–195) of Xyr1 was expressed by the pGEX system according to the manufacturer's guidelines. The first-strand cDNA was used as a template to amplify the fragment encompassing the ORF of DNA binding domain of Xyr1 using the primers indicated in Table S1. The fragment was then ligated into plasmid pGEX-4T-1 via BamHI and Xhol double digestion to produce pGEX-4T-Xyr1-Binding and subsequently introduced into E. coli BL21 (DE3) for protein production. Purification and verification of the GST-fused proteins were performed according to the methods described previously [41].

Table S1. The fragment was then ligated into plasmid pGEX-DNA binding domain of Xyr1 using the primers indicated in a template to amplify the fragment encompassing the ORF of DNA binding domain of Xyr1 (Table S1). The GST-fused Xyr1 was expressed by the pGEX system according to the methods described previously [41].

2.7. Electrophoretic Mobility Shift Assays (EMSA). A universal primer (5′-ACTAACTCAGGTCTACTG-3′) was labeled at 5′-terminal with Cyanine 5 (Cy5) (Sangon, Shanghai, China). Cy5-labeled DNA probes were generated by two steps PCR amplification using the primers as shown in Table S1: first, double-stranded DNA fragments were amplified from the genomic DNA of T. reesei Rut-C30 using specific primer pairs with universal primer sequence in their 5′-terminals; second, Cy5-tag was added to the above DNA fragments by PCR reaction using the universal primer labeled with Cy5. The resulting Cy5-labeled probes were recovered by agarose gel electrophoresis. EMSA was performed using a constant amount (10 ng) of labeled DNA probe. The purified protein of Xyr1 binding domain was preincubated with Cy5-labeled probe and then subjected to electrophoresis according to Ren et al. [42]. The gel was visualized using Starion FLA-9000 Scanner (FujiFilm, Japan).

3. Results and Discussion

3.1. Deletion of xyr1 in T. reesei Abolished Lignocellulolytic Enzyme Production. We constructed an xyr1 deletion strain (Δxyr1) by replacing the xyr1 open reading frame (ORF) with the hygromycin B resistance gene in T. reesei Rut-C30. We also constructed a xyr1 recomplementation strain (xyr1-rec) by replacing the hygromycin B resistance gene with a phleomycin resistance gene and the xyr1 gene in Δxyr1 (Table S1; Fig. S1). Rut-C30 and xyr1-rec displayed normal profiles of secreted proteins when cultured on cellulose-inducing media, whereas Δxyr1 produced significantly less detectable secreted protein under the same conditions, as shown in the polyacrylamide gel electrophoresis profile (Figure 1(a)) and the assay of extracellular protein concentrations (Figure 1(b)).

We measured the cellulase and xylanase activities of the secreted proteins from Rut-C30 and Δxyr1 under inducing conditions. The cellulase activity of Δxyr1 was almost completely abolished, whereas the secreted proteins of the parent strain Rut-C30 had an FPA (filter paper activity) of 14.4 IU ml⁻¹ (Figure 1(c)). Although the culture supernatants of Rut-C30 had a xylanase activity of 529.2 IU ml⁻¹, Δxyr1 had nearly no detectable xylanase activity (Figure 1(d)). A similar phenomenon was reported previously in T. reesei Qm9414 and its xyr1 deletion mutant [9]. The role of Xyr1 in regulating cellulase and xylanase gene expression is strain-independent.

During 7 d of cultivation on lignocelluloses, the biomass of Rut-C30 increased from 54 μg ml⁻¹ on the first day to 87 μg ml⁻¹ on the seventh day, whereas the biomass of Δxyr1 did not increase detectably (Figure 1(e)). Δxyr1 appeared to be unable to produce lignocellulolytic enzymes that hydrolyzed the lignocellulolytic substrates into monosaccharaides, for further utilization as a carbon source for mycelia growth. T. reesei Qm9414 and its Δxyr1 strain showed similar growth rates on plates containing xylan or cellulose [9]. This discrepancy might be due to the different methods used to measure growth rate. Similar to our findings, XLR-1 was previously recognized as a homolog of Xyr1/xlnR and regulated some hemicellulase gene expression in N. crassa. Deletion of xlr1 resulted in minimal growth on xylan, whereas the parental strain grew well [13].

To exclude the growth difference in measuring protein secretion, we normalized protein secretion by the biomass of Rut-C30 and Δxyr1. Extracellular protein concentration of the parent strain Rut-C30 was also significantly higher than that of Δxyr1 (Figure 1(f)). By contrast, no significant difference in growth, secretory protein concentrations, or activities of FPase and xylanase was observed between Rut-C30 and Δxyr1 cultured in medium with glucose as the sole carbon source. All values were similar to those of Δxyr1 cultured on cellulose-inducing media (Figures 1(b)–1(f)).

3.2. RNA-Seq Data Processing and FunCat Analysis. In preparation for RNA-seq, the T. reesei Rut-C30 and Δxyr1 strains were precultured in SDB (Sabouraud’s dextrose broth) for 48 h and then the mycelium was collected, washed, and transferred into medium containing lignocellulose or glucose for another 15 h, and then samples were prepared for RNA isolation and further sequencing. The 26.8–49.8 M reads generated corresponded to different samples. After sequence quality control and mapping, the number of properly paired reads per sample ranged from 13.96 to 26.56 M (Table S2). The transcription levels of 22 genes, which were expressed differentially in the parent strain Rut-C30 and its xyr1 deletion strain, according to RNA-seq data, were further analyzed by quantitative reverse-transcriptase polymerase chain reaction (Table S3). The results were consistent with the results from transcription profiling.

When cultured on lignocellulosic medium, 467 genes were expressed differentially in the Δxyr1 strain compared with the parental strain Rut-C30 (Table S4). Among these genes, 177 were found to be downregulated and the other 290 were upregulated in Δxyr1. Polysaccharide metabolism, transport, cell rescue/defense and virulence, lipid metabolism, interaction with the environment, protein fate, energy, secondary metabolism, biogenesis of cellular components, transcription, amino acid metabolism, the signal transduction mechanism, cell fate, protein synthesis, nucleotide metabolism, and aromate metabolism were the main functional categories (fine-sorted after being classified by FunCats) to which the 467 differentially expressed genes were allocated (Figure 2).

We also examined the expression of genes affected by xyr1 deletion when strains were cultured on glucose. A total of 281 genes were found to be significantly affected; 186 genes were downregulated in the Δxyr1 strain, and 95 genes displayed significantly higher expression levels in
Figure 1: Analysis of fermentation liquor of strains Rut-C30, Δxyr1, and xyr1-rec in lignocellulosic and glucose medium. (a) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of culture supernatants of Rut-C30, Δxyr1, and xyr1-rec. For each sample, 10 μl supernatant was loaded. (b) Protein concentration, (c) FPase activity, (d) xylanase activity, (e) biomass, and (f) protein concentration (normalized by biomass of samples) of Rut-C30 and Δxyr1 in lignocellulosic and glucose medium. For (b), (e), and (f), samples were collected after cultivation for 1, 2, 3, and 7 d. For SDS-PAGE (a) and characterization of FPase activity (c) and xylanase activity (d), samples collected 7 d after fermentation were used. All values presented in (b)–(f) are means of three independent measurements; error bars indicate standard deviations. *p < 0.01.
Δxyr1 (Table S5). Among the 281 genes, 47 also appeared with the genes transcriptionally affected by xyr1 deletion under induced conditions (Table S6). The main categories represented among the 186 downregulated genes were those involved in cell rescue, defense, virulence, energy, transport, lipid metabolism, secondary metabolism, amino acid metabolism, interaction with the environment, and polysaccharide metabolism (Figure 3). On the other hand, the main categories represented among the 95 upregulated genes were involved in energy, transport, amino acid metabolism, and protein fate and synthesis (Table 3; Table S5).

In the parental strain Rut-C30, 338 genes were upregulated and 741 genes were downregulated under induced (lignocellulose) compared with repressed (glucose) conditions (Table S7). Notably, there were many more genes with changed expression levels than the Δxyr1 strain under the induced and repressed conditions, which may be because (1) full activation of the function of Xyr1 required special culture conditions and (2) changing the culture conditions affected not only Xyr1, but also other regulators. Analysis of genes existing in the intersection of Rut-C30/Δxyr1 under induced conditions and the Rut-C30 (lignocellulose)/Rut-C30 (glucose) would facilitate understanding of the regulation mechanisms of these genes (Table S8).

3.3. Functional Xyr1 Stimulated the Expression of Lignocellulose Degradation-Related Genes. When cultured on lignocellulosic medium, most of the 177 downregulated genes in the Δxyr1 strain were involved in carbohydrate metabolism, compared with the parent strain Rut-C30 (Table S4). In addition to previously reported cbh1, cbh2, egl1, and bgl1 [9], expression of the other functional cellulase genes egl2, egl3, egl4 (cel61a), egl5, cel61b, and bgl2 was significantly impaired in the Δxyr1 strain (Table 1).

Additional nonenzymatic cellulose-attacking proteins were also regulated in a coordinated fashion with other cellulose-degrading enzymes (Table 1). These proteins include swollenin SWO1, which is a protein carrying a cellulose-binding domain and an expansin-like domain that disrupts the crystalline cellulose structure [43], and cip1 and cip2, which encode a CE15 glucuronylesterase [44]. Both cip1 and cip2 contain cellulose-binding domains and signal sequences [45].

A previous report indicated that Xyr1 is not involved in the activation of bgl2 (TriireRUTC30: 127115) expression [9]. Although the results from our study showed that it was significantly downregulated in the xyr1 deletion mutant, the function of cip1 remains unknown. To further determine whether Xyr1 could directly regulate bgl2 and cip1, the DNA fragments from promoter regions of bgl2 and cip1 were chosen as the candidates in electrophoretic mobility shift assays (EMSAs). Substantial gel shifts were observed for the labeled probes corresponding to the promoter regions of bgl2 and cip1 and typical protein concentration-dependent binding trends appeared (Figures 4(a) and 4(b)). The results suggest that Xyr1 activated the expression of bgl2 and cip1 directly, by binding to their promoter regions, just as it was previously reported to bind to the promoter regions of cellulase genes [15]. As each of the two promoters has more than one 5′-GGC(A/T)3-3′ motif, various Xyr1-DNA complexes could be formed, which was reflected in different low mobility bands (Figures 4(a) and 4(b)).

Similarly, deletion of xyr1 affected expression of hemicellulase genes on the inducing medium (Table 1). In T. reesei,
Table 1: Changes in CAZome gene expression between *T. reesei* Rut-C30 and Δxyr1 under the lignocellulose condition.

| Protein ID | log2 ratio Rut-C30/Δxyr1 | Q-value |
|------------|---------------------------|---------|
| **Cellulases** | | |
| Endoglucanase 1 cel7b | 5304 | 9.64323 | 0 |
| Endoglucanase 2 cel5a | 72489 | 10.4187 | 0 |
| Endoglucanase 3 cel2a | 124438 | 8.46247 | 1.07E-07 |
| Endoglucanase 5 cel45a | 25940 | 9.22161 | 2.18E-08 |
| Exoglucanase 1 cel7a | 125125 | 10.8384 | 0 |
| Exoglucanase 2 cel6a | 122470 | 10.6842 | 0 |
| Cellulase enhancing protein cel61a | 139633 | 10.1565 | 0 |
| Cellulase enhancing protein cel61b | 122518 | 10.063 | 0 |
| β-Glucosidase 1 cel3a | 136547 | 5.89937 | 0 |
| β-Glucosidase 2 cella | 127115 | 1.83786 | 3.43E-05 |
| β-Glucosidase cellb | 77989 | 2.52453 | 5.64E-11 |
| β-Glucosidase cel3d | 122693 | 4.9912 | 0 |
| β-Glucosidase cel3e | 74305 | 1.24505 | 0.002047 |
| β-Glucosidase | 109567 | −1.18554 | 0.004262 |
| **Hemicellulases** | | |
| Acetylxyylan esterase axel | 139631 | 10.4907 | 0 |
| Acetylxyylan esterase | 88887 | 8.921338 | 3.99E-08 |
| α-Galactosidase | 71638 | 4.96894 | 1.37E-08 |
| α-N-Arabinofuranosidase abf1 | 102517 | 3.19423 | 1.50E-07 |
| α-Xylosidase | 134448 | 6.166894 | 2.22E-08 |
| β-Xylosidase | 77521 | 4.332579 | 4.09E-07 |
| Endo-1,4-β-xylanase 1 xyn1 | 38418 | 3.793969 | 0.00173 |
| Endo-1,4-β-xylanase 2 xyn2 | 124931 | 10.536 | 0 |
| Endo-1,4-β-xylanase 3 xyn3 | 23616 | 10.536 | 0 |
| Mannan endo-1,6-α-mannosidase | 126869 | 1.63426 | 0.001508 |
| α-L-Arabinofuranosidase abf2 | 118070 | 6.129569 | 6.81E-07 |
| β-Galactosidase | 101346 | 2.59714 | 5.30E-11 |
| β-Mannosidase | 67432 | 5.82358 | 0 |
| Exo-1,4-β-xilosidase | 140746 | 11.148 | 0 |
| Mannan endo-1,4-β-mannosidase | 122377 | 7.256397 | 4.35E-05 |
| Xyloglucanase | 111943 | 6.34823 | 0 |
| Glucuronoxylanase xynC | 93498 | 10.5903 | 5.56E-11 |
| α-Glucuronidase | 90302 | 8.110081 | 5.93E-06 |
| **Nonenzymatic cellulose attacking enzymes** | | |
| Cip1 | 121449 | 10.417 | 0 |
| Cip2 | 125757 | 11.0829 | 0 |
| Swollenin | 104220 | 8.73037 | 0 |
| **Chitinases** | | |
| Chitinase | 94061 | −1.583 | 0.009666 |
| Chitinase | 142298 | −1.60663 | 3.00E-05 |
| Chitinase | 7503 | −1.74855 | 0.00568 |
| Endochitinase | 33168 | −1.71966 | 0.002243 |
| Endochitinase | 33886 | −2.0161 | 4.09E-07 |
| Endochitinase | 124526 | −1.52294 | 5.15E-05 |
| Endochitinase | 142123 | 2.15448 | 5.61E-08 |
| Exochitinase | 104242 | −2.13026 | 4.56E-09 |
| **Glucan 1,3-β-glucosidase** | | |
| Glucan endo-1,3-β-glucosidase | 125426 | −1.71506 | 0.0010449 |
| Glucan endo-1,3-β-glucosidase | 125427 | −2.13884 | 0.0004273 |
| Glucan 1,3-β-glucosidase | 25104 | −2.27709 | 3.96E-08 |
| Glucan endo-1,3-β-glucosidase | 103726 | −2.34806 | 2.49E-05 |
hemicellulase genes xyln1, xyln2, and bxln1 were previously reported to be transcriptionally activated by Xyr1 when the inducer was supplied [9]. According to the RNA-seq data in this study, the β-1,4-xylan main chain degrading enzyme gene xyln3 and the exo-1,4-β-xylosidase gene, which hydrolyzes D-xylose from the nonreducing end of xylan, were found to be downregulated in a coordinated fashion with xyln1, xyln2, and bxln1. Other genes encoding hemicellulases, which hydrolyze the side groups linked to the β-1,4-xylan main chain, were also reported to be activated by Xyr1. These genes included α-arabinofuranosidases (abf1 and abf2), which remove the arabinose side chain, β-galactosidases, which catalyze the hydrolysis of β-galactosidase from the side groups, β-mannosidases, which hydrolyze the β-1,4-manno-oligomers, acetylxyylan esterases, which remove an acetyl group from the xylan backbone, α-glucuronidases, which cleave the α-1,2-glycosidic bond of the 4-O-methyl-D-glucuronic acid side chain of xylans, and xyloglucanase, which releases glucose from xyloglucan [46–48]. Thus, almost all functional cellulase and hemicellulase genes in T. reesei were coregulated by the TF Xyr1, and the disruption of xyr1 severely blocked their expression under inducing conditions.

In addition, we analyzed the transcription of genes potentially involved in the xylose metabolism pathway, which may be crucial for the expression of xylanases [49]. Two genes encoding D-xylose and L-xylulose reductase were also found to be downregulated in Δxyr1 on lignocellulose-inducing media. Both D-xylose and L-xylulose reductase may participate in xylose metabolism. This result was consistent with results from previous studies [11, 26]. In T. reesei, D-xylose reductase was required to metabolize D-xylose to achieve full induction of xylanase expression [49]. The L-xylulose reductase transcript was reported to be absent in a T. reesei Δxyr1 strain, which severely affects its growth on D-xylose as the sole carbon source [9]. The expression

| Other glycoside hydrolases | Protein ID | log, ratio Rut-C30/Δxyr1 | Q-value |
|----------------------------|------------|---------------------------|---------|
| Probable exopolygalacturonase X | 91667 | 1.7976e + 308 | 9.37E – 09 |
| Glucuronoxylanase xynC | 90847 | 10.4524 | 9.37E – 09 |
| Endo-β-1,6-galactanase | 11580 | 0 | 0 |
| 1,3-β-Glucanotransferase gel4 | 103899 | 0 | 0 |
| Probable endopolygalacturonase | 133383 | 3.07815 | 6.93E – 06 |
| 1,4-α-Glucan-branching enzyme | 114146 | 0 | 0.00350323 |
| Putative endo-1,3(4)-β-glucanase 2 | 100837 | -1.52054 | 0.000245344 |
| α-1,2-Mannosidase | 122299 | -1.59948 | 3.01E – 05 |
| Glucan endo-1,3-α-glucosidase agn1 | 94877 | -3.05265 | 1.76E – 13 |

Figure 3: Significant examples of functional categories of genes differentially expressed under the glucose condition. The blue bars indicate genes downregulated, and the red bars indicated genes upregulated, in Δxyr1 compared with Rut-C30.
of xylose reductase genes in *A. niger* is also dependent on XlnR, a homolog to Xyr1 [50]. These results indicate that Xyr1 regulates xylan-decomposing and xylose-metabolizing genes, suggesting that these genes are subjected to concerted evolution (Fig. S2).

Although lignin is another main component of lignocellulose, *T. reesei* is not considered to be a potential lignin-degrading fungus [51, 52], and no ligninolytic activity has been reported. However, a recent study demonstrated that some lignin depolymerizing oxidoreductases, including laccase, glyoxal oxidase, peroxidase/catalase, L-ascorbate peroxidase, copper/zinc superoxide dismutase, and several other oxidoreductases, are expressed when *T. reesei* Rut-C30 is grown on natural lignocellulosic biomass [53].

In this study, expression levels of two laccase genes (TrireRUTC30: 104519; TrireRUTC30: 36885) were higher on lignocellulose medium than on glucose-repressing medium in Rut-C30 (Table S7). In the Δxyr1 strain, one of the laccase genes (TrireRUTC30: 104519) could also be induced on lignocellulosic medium (Table S9). However, other lignin-degrading genes, such as glyoxal oxidase and Cu/Zn superoxide dismutase (TrireRUTC30: 26844, TrireRUTC30: 112797), were not induced on lignocellulose in either strain (Tables S7 and S9). No gene encoding typical lignin-degrading enzymes, including the six laccase-like multicopper oxidase-encoding genes [51], was downregulated in Δxyr1. Although several lignin degradation-related enzymes could be induced by lignocelluloses, their expression was possibly Xyr1-independent, which inferred a specific regulatory pattern in controlling the expression of the lignin-degrading enzyme genes.

When cultured on glucose, *xyr1* deletion in Rut-C30 resulted in the downregulated expression of six genes.

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**Figure 4**: Xyr1 functionally binds to the upstream regions of four genes downregulated in Δxyr1 on inducing medium. Binding of Xyr1 to the promoter regions of the intracellular β-glucosidase-encoding gene *bgl2* (a), the nonenzymatic cellulose-attacking enzyme-encoding gene *cip1* (b), the putative MFS lactose transporter-encoding gene *lp* (c), and the *nmr*-like gene (d). The amounts of purified Xyr1 (µM) used were as indicated and about 10 ng of Cy5-labeled probes was added to each reaction. The specificity of shifts was verified by adding 100-fold excess unlabeled specific (S) and nonspecific (NS) competitor DNA. Purified GST (1 µM) was used as the negative control to exclude nonspecific binding by GST.
encoding lignocellulolytic enzymes (Table S5). For example, transcriptional levels of cbh1 and cbh2 were reduced by 5.4- and 8-fold, respectively, in Δxyr1 compared with the transcription levels in Rut-C30 (Table S5). At the protein level, the band of CBH1 could be detected in Rut-C30 and the xyr1 recombination strain xyr1-rec cultured on glucose, but not obvious in the xyr1 deletion strain Δxyr1 (Figure 1(a)). Although other lignocellulolytic enzymes did not show statistically significant differences, their expression levels decreased similarly (data not shown). These results suggest that, for the carbon catabolite derepression strain Rut-C30, Xyr1 was partially functional in controlling the expression of lignocellulolytic enzymes, even under repressed conditions.

Among the 1079 differently expressed genes (338 upregulated and 741 downregulated genes) of the parent strain Rut-C30 cultured under the induced (lignocellulose) and repressed (glucose; Table S7) conditions, a total of 69 genes (53 upregulated and 16 downregulated) were categorized as involved in carbohydrate metabolism, according to the FunCat classification (Table S10). Among the 53 upregulated genes, 35 were detected among the downregulated genes in Δxyr1 under lignocellulose conditions compared with those of the parental strain Rut-C30 (Tables 1, S8, and S10), which included almost all functional cellulose- and hemicellulose-encoding genes. These 35 putative Xyr1-regulated target genes may have been activated under the lignocellulose condition to participate in carbohydrate metabolism, while the remaining 18 genes were potentially coregulated by other unidentified regulators. By contrast, only one cellulase gene, TrireRUTC30: 109567 (BGL, GH3 family; FPKM [the number of fragments per kilobase per million fragments mapped])Δxyr1 lignocellulose: 107.295, FPKM Δxyr1 glucose: 5.3187), was detected to be expressed differently in Δxyr1 under the induced and repressed conditions.

Further in vivo or in vitro assays would be needed to detect whether any of the 53 upregulated genes were directly regulated by Xyr1. For example, bgl2 (TrireRUTC30: 127715) was not detected as upregulated in Rut-C30 under lignocellulose compared with glucose conditions. Because of these results, bgl2 was previously regarded as not regulated by Xyr1 [9]. bgl2 could also bind with Xyr1 in the gel retardation assays (Figure 4(a)). In this case, bgl2 might be subjected to the combined reactions of Xyr1 and several other coregulators.

Overall, the above results supported the global role of Xyr1 as an essential regulator in activating lignocellulose degradation-related genes, including cellulose- and hemicellulose-encoding genes and genes participating in xylose metabolism, but not lignin degradation-related genes. Nonenzymatic cellulose-attacking enzymes and two glycoside hydrolase family AA9 protein (Cel61a and Cel61b)-encoding genes harbored coordinated transcription changes with (hemi)cellulase genes, which suggested that they were under the same regulation of Xyr1 and played key roles in lignocellulose degradation. Even under glucose conditions, Xyr1 plays a partial role in regulation of the expression of cellulase genes in T. reesei Rut-C30, which implies its direct or indirect interactions with Cre1 in T. reesei wild-type strains.

3.4. Transcription Levels of Transporter Genes Affected by xyr1 Deletion in T. reesei Rut-C30. Transporter was the second largest category of genes downregulated in the Δxyr1 strain when cultured on lignocellulose, which is comprised of 21 transporter-encoding genes (Table S11; Figure 2), among which 18 genes belonged to the major facilitator superfamily (MFS). The MFS transporters are single-polypeptide secondary carriers capable of transporting only small solutes [54], and they are distributed ubiquitously throughout virtually all currently recognized organismal phyla [55]. The 18 MFS genes were classified into seven families, according to the Transporter Classification Database (http://www.tcdb.org/), which included the sugar porter (SP), fructose H+ symporter, nitrate/nitrite porter families, the phosphate H+ symporter, monocarboxylate porter, anion:cation symporter, and vacuolar basic amino acid transporter families (Figure 5(a)).

Among the 18 MFS genes, 11 genes belong to the SP family (Table S11), which is the largest MFS family transporting sugars such as glucose, fructose, mannose, galactose, xylose, maltose, lactose, α-glucoside, and quinate [56]. In addition, the expression levels of 9 of the 11 SP family members were significantly reduced in the parental strain Rut-C30, which was cultured from inducing (lignocellulose) to repressing (glucose) conditions (Tables S8 and S11; Figure 5). Our EMSA experiments, by testing the purified Xyr1 and a putative lactose transporter gene TrireRUTC30: 127980 belonging to these SP family members (Figure 4(c)), confirmed that the transcription of SP transporters might be directly regulated by Xyr1 in T. reesei or its homolog in other organisms.

A sorophorose-inducible β-diglucoside permease was previously reported to be involved in the induction of the cellulases in T. reesei [57], indicating that some sugar transporters have an important role in induction of cellulase expression. The induced expression of sugar transporters might be the previous and necessary step in the induction of cellulose-encoding genes, by promoting the transportation of inducible glucose into the cell. Galazka et al. [58] introduced the celloxtrin transporter from N. crassa into Saccharomyces cerevisiae, which has led to efficient growth of this yeast on celloextrins. However, the functions of the Xyr1-dependent transporters have not yet been characterized. Recently, one of these Xyr1-dependent transporters (TrireRUTC30: 109243, Trire2: 3405) was identified as essential for lactose uptake and cellulase induction by lactose [59]. In another study, the deletion strain of Trire2: 3405, crt1, showed severe growth defects on Avicel [60]. These results indicated that Xyr1’s role, as a major activator of cellulases, was achieved by regulating the transcription of some transporters. Likewise, II carbohydrate transporter genes were identified to depend on a functional XLR-1 for increased expression levels when exposed to xylan in N. crassa [13], indicating that the impact of Xyr1 or its homolog on the expression of transporter genes is not a phenomenon exclusive to T. reesei. As the uptake of cellulose oligosaccharides could play an important role in cellulase
formation [57], identification of the characteristics of various sugar transporters is important and would further assist in the identification of the cellulase induction mechanism.

ATP-binding cassettes (ABCs) containing ABC transporters are also reported to be important sugar transporters. Our RNA-seq data demonstrated that no ABC transporter gene was downregulated by deletion of xyr1 or by culture under the repressing condition (Tables S11; S12). However, seven genes encoding ABC transporters were upregulated after xyr1 deletion under lignocellulose-inducing conditions (Table S11; Figure 5(a)). These results implied that the ABC transporters are inconsistent for the expression trends of (hemi)cellulase genes that might be coregulated by Xyr1. By contrast, two ABC transporters (Tr 2687 and Tr 58366) were reported downregulated under the cellulose or glucose condition in the Δxyr1 mutant strain compared to its parental strain Qm9414 [16]. Another nine MFS transporter genes also exhibited increased expression levels after deletion of xyr1 under lignocellulose-inducing conditions (Table S11).

Whether the increased expression of these transporters contributed to nutrient uptake under starvation, due to an inability to utilize lignocelluloses in Δxyr1, is not known. Thus, two MFS transporter-encoding genes—the putative MFS glucose transporter rco3 (TrireRUTC30: 136988) and the predictedMFS siderophore iron transporter sit (TrireRUTC30: 115870)—were selected for EMSAs. However, no gel retardation was observed for the probes corresponding to the promoters of these two genes (Fig. S3A and B). These results suggest that the upregulated transporter genes were not regulated directly by Xyr1.

3.5. Several Genes Relevant to Basal Metabolism Were Inclined to Be Repressed by Xyr1. More genes encoding enzymes related to basal metabolism, such as lipid metabolism, protein fate, amino acid metabolism, and nucleotide metabolism,
were detected to be upregulated in \( \Delta xyr1 \) than in Rut-C30 under inducing conditions (Figure 2). For example, \( xyr1 \) deletion resulted in increased expression of 11 genes encoding enzymes participating in amino acid metabolism and 6 genes participating in nucleotide metabolism when cultured on lignocelluloses (Figure 6). In addition, eight lipase genes and eight protease genes were upregulated (five of them were predicted to be secreted using SignalP V4.0 program [http://www.cbs.dtu.dk/services/SignalP/]; Figure 6).

Chitinases participate in fungal cell-wall morphogenesis, including spore germination, hyphal elongation, hyphal branching, and autolysis of mycelium [61–63]. Members of the fungal genus \( \text{Trichoderma} \) are known to produce chitinase, but very little is known about the regulation of expression of these chitinase genes. According to our RNA-seq data, six genes (TrireRUTC30: 94061 (chi18-12), 142298, 7503 (chi18-15), 33168 (chi18-6), 124526 (chi18-5), and 104242) encoding secreted chitinases [64] were found to be upregulated after \( xyr1 \) deletion (Table 1). By contrast, the transcription levels of these genes did not differ between strains in the medium with glucose as a carbon source. In accordance with this, as Bischof et al. reported, one of the major differences in the \( T. \text{reesei} \) transcriptome between wheat straw and lactose is the wheat straw specific chitinases and mannosidase, which were significantly higher expressed in an \( xyr1 \)-deletion strain [65]. Seidl et al. found that chi18-12 belonged to Group B, chi18-6, and chi18-5 belonged to Group A, while chi18-15 did not belong to any group. In this case, we could predicted that TrireRUTC30: 94061, 33168, and 124526 were Chitinases possibly involved in mycoparasitism [64]. Induction of chitinase genes could be influenced not only by the presence of chitin, but also by carbon catabolite repression, the N source, and starvation [66]. As the strain \( \Delta xyr1 \) might be subjected to a shortage of nutrients, as reflected in retarded mycelia growth (Figure 1(e)), these chitinase genes seemed to be induced by carbon starvation.

One of the upregulated putative chitinase genes, \( \text{endo} \ T \) (TrireRUTC30: 142298), was shown to be not involved in chitin degradation, but it has mannosyl-glycoprotein endo-\( N \)-acetyl-\( \beta \)-D-glucosaminidase activity [67]. It was reported to be responsible for \( N \)-deglycosylation of proteins expressed and secreted by \( T. \text{reesei} \) [68]. According to our RNA-seq data, the \( \text{endo} \ T \) in Rut-C30 was significantly upregulated under lignocellulose conditions (FPKM = 42.87) compared with glucose conditions (FPKM = 3.74). However, \( \text{endo} \ T \) further displayed significantly increased expression levels in the \( xyr1 \) deletion mutant \( \Delta xyr1 \) compared with Rut-C30 when cultured on lignocelluloses, with FPKM values of 130.57 and 42.87, respectively. These controversial results suggest that the regulatory mechanism of \( \text{endo} \ T \) is very complex.

Foreman et al. [45] reported that \( \text{endo} \ T \) is not coregulated with the expression of cellulase genes in \( T. \text{reesei} \) Qm6a or RL-P37. Whether the increased expression of \( \text{endo} \ T \) was in response to carbon starvation remains unknown, as does the mechanism of regulation by Xyr1. As a result, EMSAs were performed and strong gel retardation was observed for the probe corresponding to the upstream region of the putative \( \text{endo} \ T \) with Xyr1 (Figure 7(a)). In \( T. \text{reesei} \) RutC-30, the expression of \( \text{endo} \ T \) was potentially activated by Xyr1, as well as lignocellulose degradation-related genes, when cultured on lignocellulose. Thus, the resulted single GlcNAc on the heterologous expressed TrBglS benefitted from its enzymatic activity and thermostability, compared with PpBglS [69]. After deletion of \( xyr1 \), the upregulation of \( \text{endo} \ T \) might be due to starvation of carbon sources, which might be an effort to deglycosylate the glycan coat of the glycoprotein composed of the cell wall, and contribute to further protease degradation [67].

In filamentous fungi, extracellular proteases are usually produced in response to carbon or N derepression [70]. These phenomena mean that genes related to nonpreferred N source utilization were stimulated in the \( \Delta xyr1 \) strain,
possibly due to the inability to utilize lignocelluloses. Among the five upregulated secreted proteases, the pepA (TrireRUTC30: 104564)-encoding putative acid aspergillopepsin I, but not the tryp (TrireRUTC30: 94189)-encoding putative trypsin with an optimal operating pH of approximately 7.5–8.5, was a putative Xyr1 target in Rut-C30 (Figures 7(b) and S3C). These results imply that Xyr1 also tends to repress the acid peptidases directly to reduce the degradation of cellulases in medium with a pH value of about 5. In addition, an α-1,2-mannosidase gene mds1 (TrireRUTC30: 122299), which participated in N-glycosylation modification of glycoprotein, showed increased expression levels in Δxyr1 (FPKM values ranged from 31.51 in Rut-C30 to 95.51 in Δxyr1). The α-1,2-mannosidase was reported to readily convert Man₃GlcNAc₂, or a mixture of Man₆₋₉GlcNAc₂ oligosaccharides, to the respective Man₃ structures [71], and was suggested to be localized in the Golgi apparatus [72]. No interaction between Xyr1 and the promoter region of this α-1,2-mannosidase gene mds1 was detected in EMSAs (Fig. S3D). In this case, the upregulated α-1,2-mannosidase expression level in Δxyr1 might be attributed largely to its carbon starvation status.

3.6. Transcription of Genes Related to Energy Metabolism.

In our study, three genes predicted to be alcohol dehydrogenase genes (TrireRUTC30: 128036, TrireRUTC30: 26479, and TrireRUTC30: 133809) were also upregulated when xyr1 was deleted in the lignocellulose condition (Table S4). In
the glucose condition, however, their expression exhibited no significant difference between Rut-C30 and Δxyr1 (Table S5). In Aspergillus nidulans, an alcohol dehydrogenase (ADHIII) was previously shown to be induced by carbon starvation stress [73]. We speculated that the transcription of these alcohol dehydrogenase genes could be sensitive to carbon starvation, but with no relationship to the existence of Xyr1. In agreement with this speculation, the expression of TrireRUTC30: 133809 was upregulated significantly in Rut-C30, as well as the xyr1 deletion strain Δxyr1, under induced conditions compared with repressed conditions (Tables S7, S9).

The expression of TrireRUTC30: 26479 was also stimulated in Δxyr1 when transferred from the glucose to the lignocellulose condition (Table S9). In addition, Trire-RUTC30: 87029, the Podospora anserine PaATG1 ortholog, was upregulated in Δxyr1 compared with Rut-C30 under a lignocellulose condition (Table S4). TrireRUTC30: 87029 is a serine-threonine kinase composed of an N-terminal kinase domain and a C-terminal domain with unknown function. The PaATG1 mutant displayed development defects characteristic of abrogated autophagy in P. anserine [74]. Autophagy is a process of protein and organelle degradation by the vacuole (lysosome). This process is conserved in organisms, and functions as a cell survival mechanism during nutrient starvation. Therefore, elevated ATG1 gene expression probably contributed to accommodating the Δxyr1 strain during carbon starvation, reflecting an xyr1 deletion that totally eliminated the capability to utilize lignocelluloses as a carbon source.

The glycolysis and citric acid cycles are among the most important energy-releasing pathways in glucose metabolism. Comparing the gene expression levels between Rut-C30 and Δxyr1 under a glucose condition, deletion of xyr1 led to significant variation in a succinyl-CoA ligase gene (Trire-RUTC30: 135123) and a glycerol-3-phosphate dehydrogenase gene (TrireRUTC30: 116453; Table S5). The AD-lactate dehydrogenase (TrireRUTC30: 73249) and NAD-dependent malic enzyme (TrireRUTC30: 69465) genes (Table S5) were also influenced by xyr1 deletion, and these two enzymes were involved in yielding the important carbohydrate metabolic intermediate pyruvate. The binding sites of Xyr1 were detected in the promoter regions of the genes mentioned above in xyr1 deletion mutants. However, more investigation is required to confirm whether Xyr1 also participates in the glucose metabolism and energy-releasing pathways.

3.7. Transcription Changes of Putative Heat Shock Proteins.
Heat shock proteins are often induced when organisms respond to extreme temperature and other stresses, such as starvation [75, 76]. Based on transcription profiling of Rut-C30 and Δxyr1 under induced and repressed conditions, we attempted to explain the roles that heat shock proteins play in lignocellulose degradation, as well as their transcriptional regulation mechanism.

The transformation of hsp23 (small heat shock protein-encoding gene of Trichoderma virens) into Trichoderma harzianum was reported to confer thermosterlance and result in higher biomass accumulation under thermal stress [77]. Under lignocellulose-inducing conditions, a gene homologous to hsp23, TrireRUTC30: 122251, was upregulated in Δxyr1 relative to its parent strain (Table S4). These results illustrate the process of lignocellulose degradation by T. reesei Rut-C30, in which the heat shock protein-encoding genes harbored expression profiles similar to profiles of the lignocellulose degradation-related genes.

Due to the putative Xyr1-binding consensuses in the upstream region of this hsp23 homolog, EMSAs were performed. The results indicated that Xyr1 could bind to the probe corresponding to the upstream region of this hsp23 homolog (Figure 7(c)). In this case, we speculated that, under induced conditions, Xyr1 was inclined to repress the expression of heat shock proteins to maintain the balance of the parent strain Rut-C30 and to ensure maximum lignocellulose degradation. In agreement with these results, even under the glucose condition, deletion of xyr1 caused upregulation of a heat shock protein Hsp78-encoding gene (TrireRUTC30: 73724; Table S5). However, another heat shock protein Hsp70-coding gene, TrireRUTC30: 25176, was downregulated in Δxyr1, suggesting that the regulation of heat shock proteins is much more complex in Rut-C30 under the glucose condition. Two putative heat shock protein-encoding genes, Hsp70 (TrireRUTC30: 97499) and Dnaj (Hsp40, TrireRUTC30: 137482), were downregulated in Rut-C30 under the lignocellulose condition compared with the glucose condition (Table S7).

3.8. Expression Profiles of Transcription Factors. With the exception of genes participating in lignocellulose degradation and transporters, the expression profiles of characterized TF genes were analyzed. We found that the Ace3 (Trire-RUTC30: 98455)-encoding gene (FPKM = 137.3–68.3) and the gene encoding the NmrA-like family domain-containing protein (TrireRUTC30: 121828; FPKM = 27.84–5.88) were also down-regulated in Δxyr1 compared with the parent strain under a lignocellulose-inducing condition (Table S4).

Ace3 is a recently identified transcription activator of cellulase genes [78]. In Δxyr1, its transcription level decreased by about 50%. Häkkinen et al. [78] reported that the expression level of xyr1 was lower in the ace3 deletion strain than in the parent strain Qm9414. In this case, Xyr1 and Ace3 might be cross-regulated, which requires further study by protein-DNA and protein-protein interaction assays. In T. reesei, Kap8 was identified to be essential for the nuclear transport of the Xyr1, and deletion of kap8 completely abolished the transcription of 42 CAzymes (including all the cellulases and hemicellulases), which resemble the phenomenon of a xyr1 loss-of-function mutant [79]. However, the transcription of xyr1 itself is not affected by kap8 deletion, implying that its transcription is hardly dependent on Xyr1-autoregulation. On the contrary, Ace3 exhibited significant reduced expression level after kap8 deletion under the induced condition, indicated that its expression is partially Xyr1-dependent [79].

NmrA is a negative transcriptional regulator involved in N metabolite repression. In A. nidulans, deletion of nmrA resulted in partial derepression of activities, with utilization of nonpreferred N sources in the case of N repression [80]. This phenomenon was also observed in other filamentous
Table 2: The transcription of several identified TFs in Rut-C30 under the induced compared with repressed condition.

| Encoding genes of TFs | Protein ID | TrirERUTC30 | FPKM (glucose) | FPKM (lignocellulose) | Significant |
|-----------------------|------------|--------------|----------------|-----------------------|-------------|
| xyr1                  | 98788      | 43.73        | 42.93          | No                    |
| cre1-1                | 23706      | 498.07       | 802.49         | No                    |
| ace1                  | 122363     | 337.92       | 21.07          | No                    |
| ace2                  | 32395      | 23.7         | 21.07          | No                    |
| ace3                  | 98455      | 81.56        | 137.33         | No                    |
| bglR                  | 91236      | 225.27       | 128.07         | No                    |
| hap2                  | 93466      | 39.57        | 21.40          | No                    |
| hap3                  | 24298      | 71.16        | 75.36          | No                    |
| clr-1                 | 68701      | 11.14        | 9.83           | No                    |
| clr-2                 | 76250      | 3.21         | 2.80           | No                    |
| nmrA                  | 121828     | 21.5         | 27.85          | No                    |
| areA                  | 140814     | 52.95        | 36.58          | No                    |

fungi, such as N. crassa [81]. We speculated that the decreased expression of this nmrA-like gene might facilitate the utilization of an N source in response to carbon starvation in the xyr1-deleted strain or be directly regulated by Xyr1. EMSA was employed to detect the potential interaction between Xyr1 and this nmrA-like gene, and a strong gel shift of the probe was observed when Xyr1 was added to the reaction (Figure 4(d)). The results suggested that, under a lignocellulose-inducing condition, Xyr1 activates the expression of nmrA to repress utilization of nonpreferred N sources, possibly through repression of the activity of the A. nidulans AreA [82] homolog in T. reesei Rut-C30, and ensure degradation of lignocellulose. However, the precise characteristic of this gene product remains to be determined.

Similar to the effect of xyr1 deletion on the expression of the nmrA-like gene when cultured on lignocellulose, Δxyr1 showed a significantly lower level of the nmrA-like gene when both strains were cultured on glucose. Obviously, decreased expression of this nmrA-like gene was not a response to carbon starvation. As described in the gel-retardation assay, the nmrA-like gene was a putative target of Xyr1 (Figure 4(d)). The results further imply that the nmrA-like gene was a putative target downstream of Xyr1 and repressed by Xyr1, regardless of the culture conditions.

The transcription levels of genes encoding transcription regulators for cellulase or hemicellulase genes were also compared in Rut-C30, under induced and repressed conditions. These genes included cre1-1, ace1, ace2, ace3, bglR, hap2, hap3, clr-1, and clr-2. However, none of these genes showed a significant response (Table 2). These results imply that all of these genes had constitutive expression and were not induced when transferred to lignocellulose-induced conditions. The two transcription regulators involved in N metabolism, nmrA-like and areA, were also analyzed and the same expression mode was detected (Table 2), suggesting that complex posttranslational regulation, such as phosphorylation on Cre1 [83], acted on these transcription regulators when T. reesei Rut-C30 was transferred from repressed to induced conditions. Considering the lack of a significant difference in xyr1 transcription levels of Rut-C30 under induced (FPKM = 42.93) and repressed (FPKM = 43.73) conditions, these results imply that the function of Xyr1 was severely repressed under repressive culture conditions.

3.9. Putative Regulation Mechanism of Xyr1 in T. reesei. As the 5′′-GGC(A/T)₃′-3′ and 5′′-GGC(A/T)₄′-3′ motifs play important roles as functional Xyr1-binding sites in T. reesei [15], we searched for these motifs in the 1-kb 5′-upstream regions of all genes. We identified an obvious tendency of increasing occurrence of Xyr1 binding sites in the upstream regions of Xyr1-upregulated genes. We analyzed the same expression mode was detected (Table 2), suggesting that complex posttranslational regulation, such as phosphorylation on Cre1 [83], acted on these transcription regulators when T. reesei Rut-C30 was transferred from repressed to induced conditions. Considering the lack of a significant difference in xyr1 transcription levels of Rut-C30 under induced (FPKM = 42.93) and repressed (FPKM = 43.73) conditions, these results imply that the function of Xyr1 was severely repressed under repressive culture conditions.

![Figure 8: Frequencies of appearance of 5′′-GGC(A/T)₃′-3′ and 5′′-GGC(A/T)₄′-3′ motifs in the upstream regions of Xyr1-upregulated genes.](image-url)
Non-enzymatic cellulose degradation genes

MFS sugar transporter -encoding genes

(hemi)cellulase genes
(including bg12)

Mainly genes involved in primary metabolism

NmrA

Figure 9: The predicted model of Xyr1-mediated gene regulation in T. reesei Rut-C30 under lignocellulose-inducing conditions. Xyr1 plays pleiotropic regulatory roles in the process of lignocellulose degradation and may be involved in primary metabolism. It is also implicated in interaction with the nonpreferred N source degradation repressor NmrA. → positive transcriptional control; ⊣ negative transcriptional control; → indirect regulation.

5'-GGC(A/T)3-3' motifs displayed an increasing frequency of appearance, from 8.6 to 10.9, corresponding to the top 50 and top 10 Xyr1-upregulated genes, respectively.

In a previous study [15], the frequency of appearance of the 5'-GGC(A/T)3-3' motifs in the 16 Xyr1-regulated genes was compared with those of all other annotated and predicted ORFs in the T. reesei genome database. The 5'-GGC(A/T)3-3' motifs were present at a higher frequency in the Xyr1-upregulated genes. This finding, combined with ours, suggests the involvement of motifs in Xyr1-mediated gene expression. However, Xyr1-downregulated genes, such as pepA and hsp23, cultured on lignocellulose showed no such frequency of motif appearance in their promoter regions (data not shown). In this case, the mechanism of Xyr1 in fine-tuning the expression of these genes requires further study.

4. Conclusions

In T. reesei, all ever-identified (hemi)cellulase genes, the intracellular bg12, genes encoding nonenzymatic cellulose-attacking enzymes, and some MFS transporter genes (most belonging to the SP family) responsible for transporting glucose, fructose, mannose, galactose, xylose, maltose, lactose, α-glucosides, and quinate, were activated by Xyr1 under the lignocellulose condition. Transcription levels of most of these Xyr1 targets were significantly decreased in the parent strain Rut-C30 cultured under the inducing (lignocellulose) condition compared with the repressing (glucose) condition.

The genes encoding TFs NmrA and Ace3, Endo T (TrireRUTC30: 142298) with mannosyl-glycoprotein endo-N-acetyl-β-D-glucosaminidase activity and the acid peptidase pepA, as well as a small heat shock protein-encoding gene hsp23, were putative Xyr1 targets. All of these transcriptional regulations might contribute to the production of (hemi)cellulases and ensure the digestion of lignocellulose to the largest extent (Figure 9). On the other hand, the transcription levels of most genes relevant to basal and energy metabolism were potentially affected by the starvation of carbon sources.

Furthermore, deletion of xyr1 also affected various genes, even when cultured on glucose, indicating that Xyr1 has pleiotropic functions in biological processes. In this study,
lignocellulose-inducing conditions do not induce expression level of \( \text{xyr1} \) but activate its function, possibly through posttranslational modifications. These findings not only improve our understanding of the regulatory roles of Xyr1, including lignocellulose degradation, transport, and interaction with N metabolism, but also provide useful information for further exploration of genes involved in lignocellulose degradation in \( T. \text{reesei} \).

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| SP | Sugar porter |
| MFS | Major facilitator superfamily |
| EMSA | Electrophoretic mobility shift assay |
| TF | Transcription factor |
| RNA-seq | RNA sequencing |
| FPKM | The number of fragments per kilobase per million fragments mapped |
| FPA | Filter paper activity |
| ORF | Open reading frame |
| ABC | ATP-binding cassette |
| qRT-PCR | Quantitative real-time PCR |
| SDB | Sabouraud’s dextrose broth |
| PDA | Potato-dextrose agar |
| N | Nitrogen |

**Disclosure**

The RNA-seq data discussed in this study have been deposited in the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/Traces/sra) with the accession number SRP028306. All the datasets supporting the results and conclusions of this article are included within the article and the additional files.

**Competing Interests**

The authors declare that they have no competing interests.

**Authors’ Contributions**

Liang Ma constructed the \( \Delta \text{xyr1} \) and \( \text{xyr1-rec} \) mutant strains, prepared the RNA samples, and drafted the manuscript. Ling Chen performed electrophoretic mobility shift assays and prepared the figures. Lei Zhang analyzed the transcription profiling data and performed the annotations and statistics. Gen Zou carried out real-time PCR analysis. Rui Liu and Yanping Jiang performed the FunCat analysis. Zhihua Zhou designed the study and revised the manuscript. All authors read and approved the final manuscript. Liang Ma and Ling Chen contributed equally to this work.

**Acknowledgments**

This work was financially supported by the National Natural Science Foundation of China (31300073 and 31470201) and High-Tech Research and Development Program of China (863:2013AA102806).

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