

Research Article

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

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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*. 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 ($\Delta xyr1$), cultured on lignocellulose or glucose. *xyr1* 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 $\Delta xyr1$. By contrast, all differentially expressed protease, lipase, chitinase, some ATP-binding cassette transporters, and heat shock protein-encoding genes were upregulated in $\Delta xyr1$. When cultured on glucose, a total of 281 genes were expressed differentially in $\Delta xyr1$, 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 cellulose 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 Cre1 [7]. Xyr1, a homolog to XlnR in *Aspergillus niger*, is a zinc binuclear cluster protein binding to a 5'-GGCTAA-3' 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 *bgl1* [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 (*bga1*) [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/xlr1/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)₃-3' motif [15], suggest that Xyr1 behaves as a pleiotropic regulator in *T. reesei*.

Recently, transcription profiling of the *T. reesei* Qm 9414 and its Δ *xyr1* 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 *cre1* [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 Δ *xyr1* was constructed as following. Plasmids were propagated in *Escherichia coli* DH5 α . 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 μ g 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 HygxhoI and Orfhyg. 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 *Bam*HI and *Xho*I and ligated into the *Bam*HI and *Xho*I digested pCambia1300, resulting in pPH. Then the 1.3 kb fragment for 5' region of *xyr1* was amplified with primers *xyr1*xhf and *xyr1*xhr. After digestion by *Xho*I, it was inserted to the *Xho*I digested pPH to generate pPH5X. At last, the 3' region of *xyr1* was amplified with primers *xyr1*bam and *xyr1*sal and digested by *Bam*HI and *Sal*I 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* recomplementation 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 Txr5Xho and Txr3Xho (Table S1). The PCR products were digested by *Bam*HI and *Xho*I, respectively, and then inserted into corresponding site of the pPB to yield pPBReX. The transformation into Δ *xyr1* strain was performed as described above and transformants were selected on PDA containing phleomycin (4 μ g ml⁻¹) as selection agent. The homologous integration of pPBReX at the *xyr1* locus of the Δ *xyr1* 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 μ l 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 the reads were filtrated when more than 20% of bases with PHRED score were lower than 30. The cleaned RNA-seq reads were mapped to the *T. reesei* Rut-C30 genome using Tophat V1.3.2 [27] with Bowtie V0.12.7.0 [28] with a minimum intron size of 20 bp and a maximum intron size of 100,000 bp; the mean inner distance between mate pairs and the standard deviation for the distribution on inner distances between mate pairs were set to 150 and 75, respectively. Transcript abundance indicated as the number of fragments per kilobase per million fragments mapped (FPKM) was estimated with the program Cuffdiff from the package Cufflinks v0.83 [29]. Differential expression values were determined using DEseq [30, 31]. FDR (False Discovery Rate) control (the cutoff was 0.01) was used to identify the significance of differences for multiple tests [32]. The genome sequence and gene structure information for *T. reesei* QM6a (<http://genome.jgi.doe.gov/Trire2/Trire2.home.html>) and Rut-C30 (<http://genome.jgi.doe.gov/TrireRUTC30.1/TrireRUTC30.1.home.html>) were obtained from the JGI genome Portal site [18, 33]. The FunCat [34] annotation for QM6a was downloaded from the PENDANT genome database [35]. The FunCat annotation for Rut-C30 was performed using a blastn [36] search between QM6a and Rut-C30. The differentially expressed genes were further fine-sorted after being classified according to their FunCat annotations.

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 *xyl1* deletion strain and *xyl1* recomplementation transformant were subjected to SDS-PAGE. 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 (Thermo electron, Finland).

For enzymatic activity measurement, the culture supernatants from the parent strain and different transformants were prepared for filter paper assay (FPA) which has been widely used to determine the total cellulase activity secreted by fungi [37]. Filter paper activity was measured according to the absorbance at 540 nm [38] in 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.

For fungal growth determination, the mycelia of different samples were used. And the biomass assays were performed as Zhao et al. reported [40] with some modifications. Briefly, the mycelia contained in 500 µl samples were collected and washed by distilled water twice and transferred to a new 1.5 ml EP tube, suspended by 100 µl distilled water. Then 1 ml of diphenylamine reagent was added into the tube, vortex thoroughly, and reaction in 60°C for 1 hour. After centrifugation for 10 min at 10000g, 200 µl supernatant was transferred to the ELISA plate and the absorbance at 595 nm was measured with a Varioskan Flash microplate reader. The biomass was indicated by the amount of DNA (mg) per ml sample. Three independent measurements were performed for all quantification assays and the data are averages of the three independent determinations. Student's two-tailed *t*-test was performed using Excel 2007 (Microsoft, WA).

2.4. Quantitative Real-Time PCR (qRT-PCR). The RNA samples obtained from *T. reesei* Rut-C30 and $\Delta xyl1$ 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 carried out on a Mastercycler (Eppendorf, Hamburg, Germany) with twin.tech real-time PCR plate 96 (Eppendorf) and Masterclear 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 denature 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 Xyl1 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)₃-3' motifs and 5'-GGC(A/T)₄-3' 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 *Bam*HI and *Xho*I 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].

2.7. Electrophoretic Mobility Shift Assays (EMSAs). A universal primer (5'-ACTAACTCGCGTACTG-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 ($\Delta xyr1$) by replacing the *xyr1* open reading frame (ORF) with the hygromycin B resistance gene in *T. reesei* Rut-C30. We also constructed an *xyr1* complementation strain (*xyr1-rec*) by replacing the hygromycin B resistance gene with a phleomycin resistance gene and the *xyr1* gene in $\Delta xyr1$ (Table S1; Fig. S1). Rut-C30 and *xyr1-rec* displayed normal profiles of secreted proteins when cultured on cellulose-inducing media, whereas $\Delta xyr1$ 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 $\Delta xyr1$ under inducing conditions. The cellulase activity of $\Delta xyr1$ 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, $\Delta xyr1$ 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 μ g ml⁻¹ on the first day to

87 μ g ml⁻¹ on the seventh day, whereas the biomass of $\Delta xyr1$ did not increase detectably (Figure 1(e)). $\Delta xyr1$ appeared to be unable to produce lignocellulolytic enzymes that hydrolyzed the lignocellulolytic substrates into monosaccharides, for further utilization as a carbon source for mycelia growth. *T. reesei* Qm9414 and its $\Delta xyr1$ 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 $\Delta xyr1$. Extracellular protein concentration of the parent strain Rut-C30 was also significantly higher than that of $\Delta xyr1$ (Figure 1(f)). By contrast, no significant difference in growth, secretive protein concentrations, or activities of FPase and xylanase was observed between Rut-C30 and $\Delta xyr1$ cultured in medium with glucose as the sole carbon source. All values were similar to those of $\Delta xyr1$ 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 $\Delta xyr1$ 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 differently 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 $\Delta xyr1$ 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 $\Delta xyr1$. 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 $\Delta xyr1$ strain, and 95 genes displayed significantly higher expression levels in

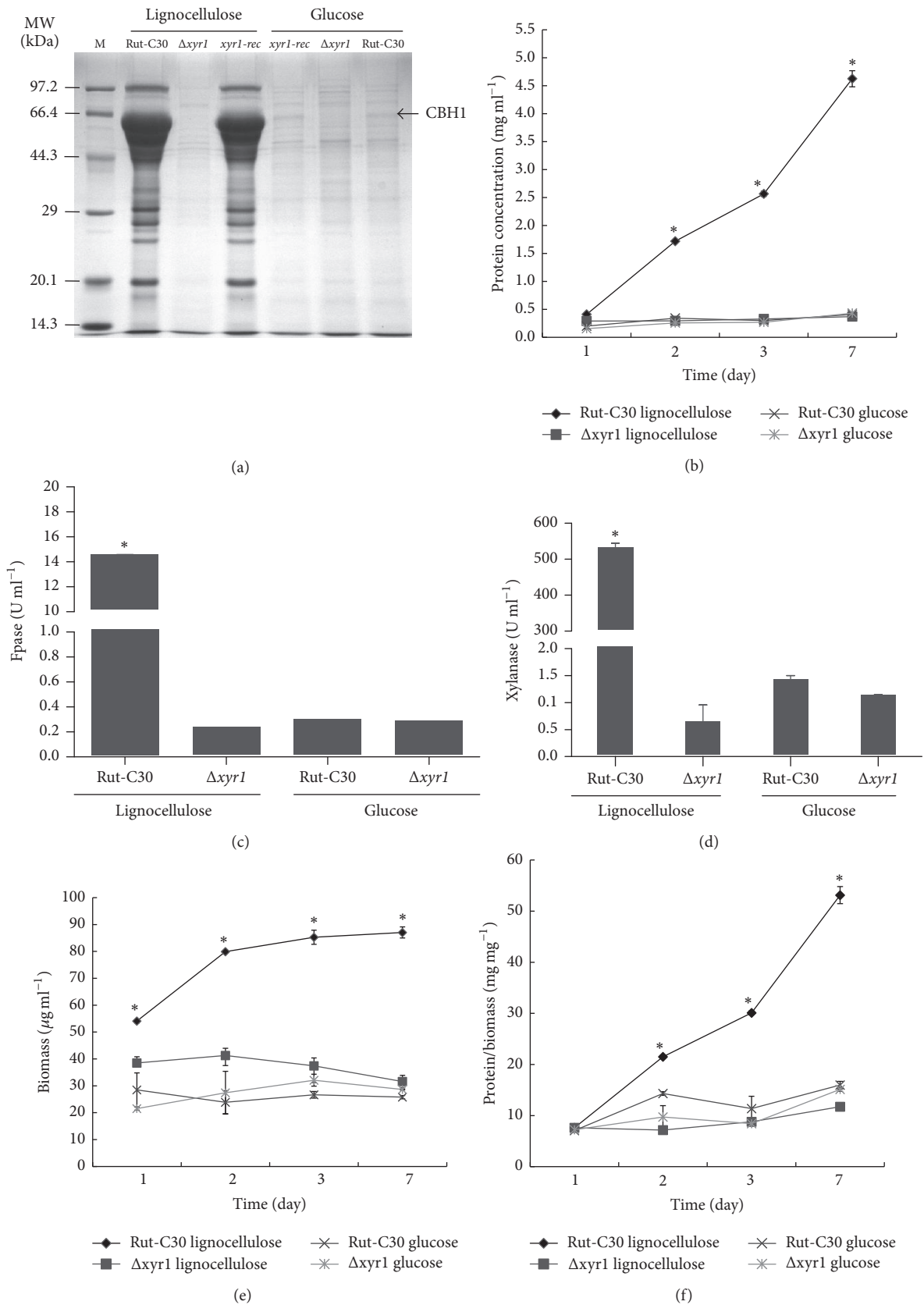


FIGURE 1: Analysis of fermentation liquor of strains Rut-C30, $\Delta xyr1$, and $xyr1-rec$ in lignocellulosic and glucose medium. (a) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of culture supernatants of Rut-C30, $\Delta xyr1$, and $xyr1-rec$. For each sample, 10 μ l supernatant was loaded. (b) Protein concentration, (c) FPase activity, (d) xylanase activity, (e) biomass, and (f) protein concentration (normalized by biomass of samples) of Rut-C30 and $\Delta xyr1$ 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. * $P < 0.01$.

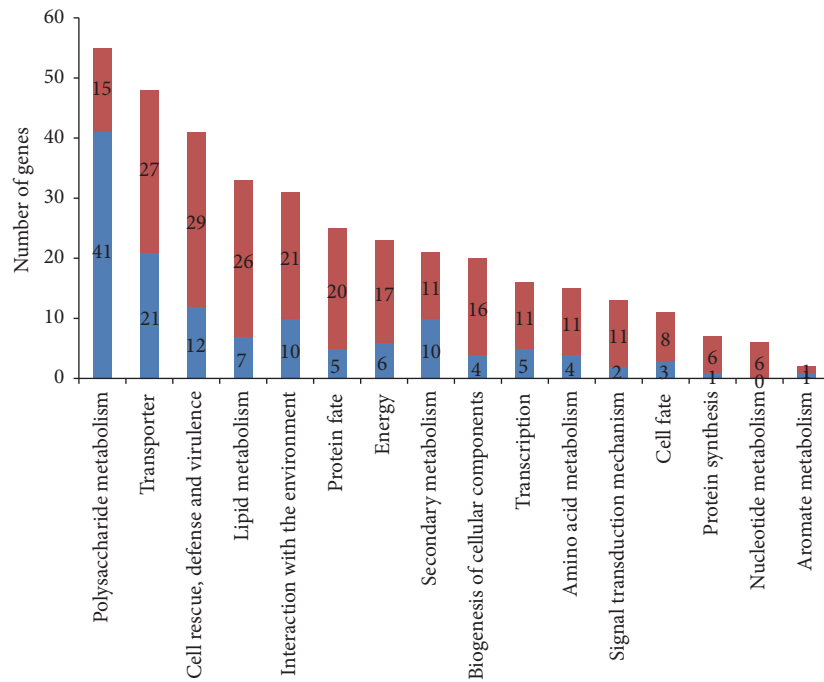


FIGURE 2: Significant examples of functional categories of genes differentially expressed under the lignocellulose condition. The blue bars indicate genes downregulated, and the red bars indicate genes upregulated, in the *xyr1* deletion strain $\Delta xyr1$ compared with Rut-C30.

$\Delta xyr1$ (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, energy, 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 (Figure 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 $\Delta xyr1$ 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/ $\Delta xyr1$ 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 $\Delta xyr1$ 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 $\Delta xyr1$ 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 glucuronyl esterase [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* (TriREUTC30: 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 $\Delta xyr1$ under the lignocellulose condition.

	Protein ID	\log_2 ratio Rut-C30/ $\Delta xyr1$	Q-value
Cellulases			
Endoglucanase 1 cel7b	5304	9.64332	0
Endoglucanase 2 cel5a	72489	10.4187	0
Endoglucanase 3 cel12a	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 cel1b	77989	2.52453	5.64E – 11
β -Glucosidase cel3d	122639	4.9912	0
β -Glucosidase cel3e	74305	1.24505	0.002047
β -Glucosidase	109567	-1.18554	0.004262
Hemicellulases			
Acetylxylan esterase axel	139631	10.4907	0
Acetylxylan 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	0.000173
Endo-1,4- β -xylanase 2 xyn2	124931	10.536	0
Endo-1,4- β -xylanase 3 xyn3	23616	10.5316	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 -xylosidase	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 – 6
Nonenzymatic cellulose attacking enzymes			
Cip1	121449	10.417	0
Cip2	125575	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	0.00101449
Glucan endo-1,3- β -glucosidase	125427	-2.13884	0.00104273
Glucan 1,3- β -glucosidase	25104	-2.27709	3.96E – 08
Glucan endo-1,3- β -glucosidase	103726	-2.34806	2.49E – 05

TABLE I: Continued.

	Protein ID	\log_2 ratio Rut-C30/ $\Delta xyr1$	Q-value
Other glycoside hydrolases			
Probable exopolygalacturonase X	91667	1.79769e + 308	9.37E - 09
Glucuronoxylanase xynC	90847	10.4524	0
Endo- β -1,6-galactanase	11580	4.68839	4.37E - 09
1,3- β -Glucanosyltransferase gel4	103899	3.38036	0
Probable endopolygalacturonase	133383	3.07815	6.93E - 06
1,4- α -Glucan-branching enzyme	114146	1.22399	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

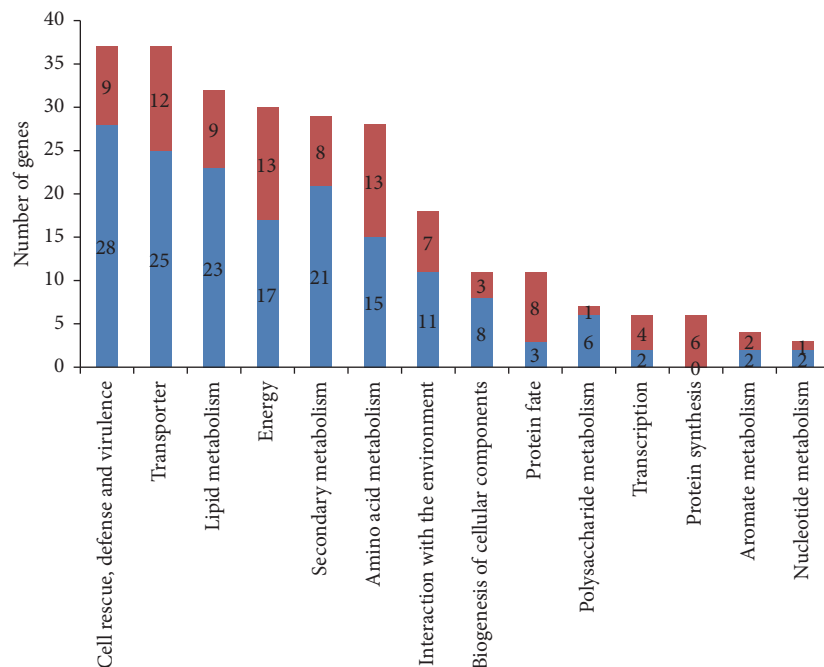


FIGURE 3: Significant examples of functional categories of genes differentially expressed under the glucose condition. The blue bars indicate genes downregulated, and the red bars indicated genes upregulated, in $\Delta xyr1$ compared with Rut-C30.

hemicellulase genes *xyn1*, *xyn2*, and *bxl1* 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 *xyn3* 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 *xyn1*, *xyn2*, and *bxl1*. 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, acetylxylan 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 $\Delta xyr1$ 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* $\Delta xyr1$ strain, which severely affects its growth on D-xylose as the sole carbon source [9]. The expression

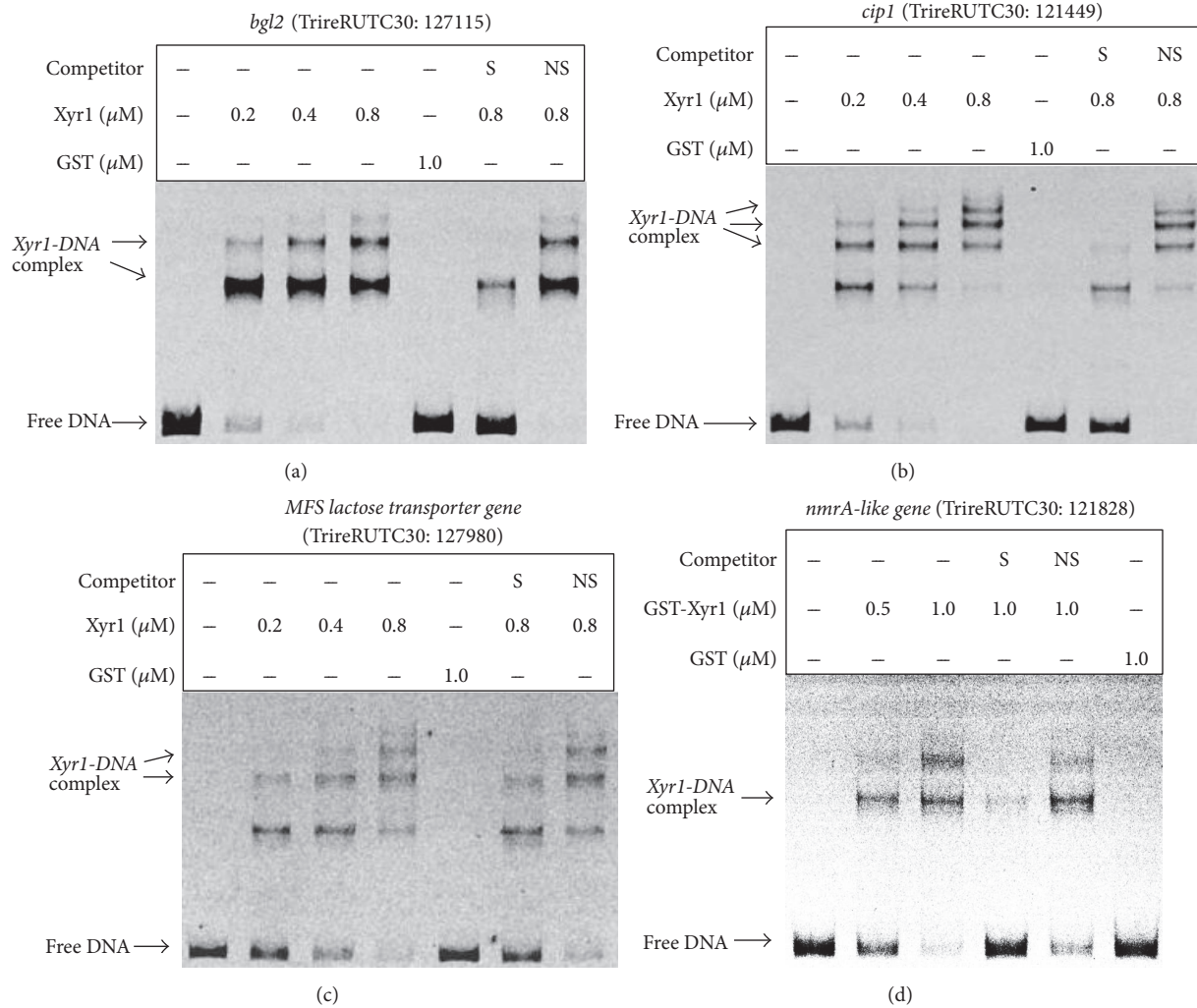


FIGURE 4: Xyr1 functionally binds to the upstream regions of four genes downregulated in $\Delta xyr1$ on inducing medium. Binding of Xyr1 to the promoter regions of the intracellular β -glucosidase-encoding gene *bgl2* (a), the nonenzymatic cellulose-attacking enzyme-encoding gene *cip1* (b), the putative 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.

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 $\Delta xyr1$ 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 $\Delta xyr1$. 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

encoding lignocellulolytic enzymes (Table S5). For example, transcriptional levels of *cbh1* and *cbh2* were reduced by 5.4- and 8-fold, respectively, in $\Delta xyr1$ 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* recomplementation strain *xyr1-rec* cultured on glucose, but not obvious in the *xyr1* deletion strain $\Delta xyr1$ (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 $\Delta xyr1$ under lignocellulose conditions compared with those of the parental strain Rut-C30 (Tables 1, S8, and S10), which included almost all functional cellulase and hemicellulase 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] $_{\Delta xyr1}$ lignocellulose: 107.295, FPKM $_{\Delta xyr1}$ glucose: 5.3187), was detected to be expressed differently in $\Delta xyr1$ 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: 127115*) 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 cellulase- and hemicellulase-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 $\Delta xyr1$ 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 sophorose-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 cellulase-encoding genes, by promoting the transportation of inducible glucose into the cell. Galazka et al. [58] introduced the cellodextrin transporter from *N. crassa* into *Saccharomyces cerevisiae*, which has led to efficient growth of this yeast on cellodextrins. 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, 11 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

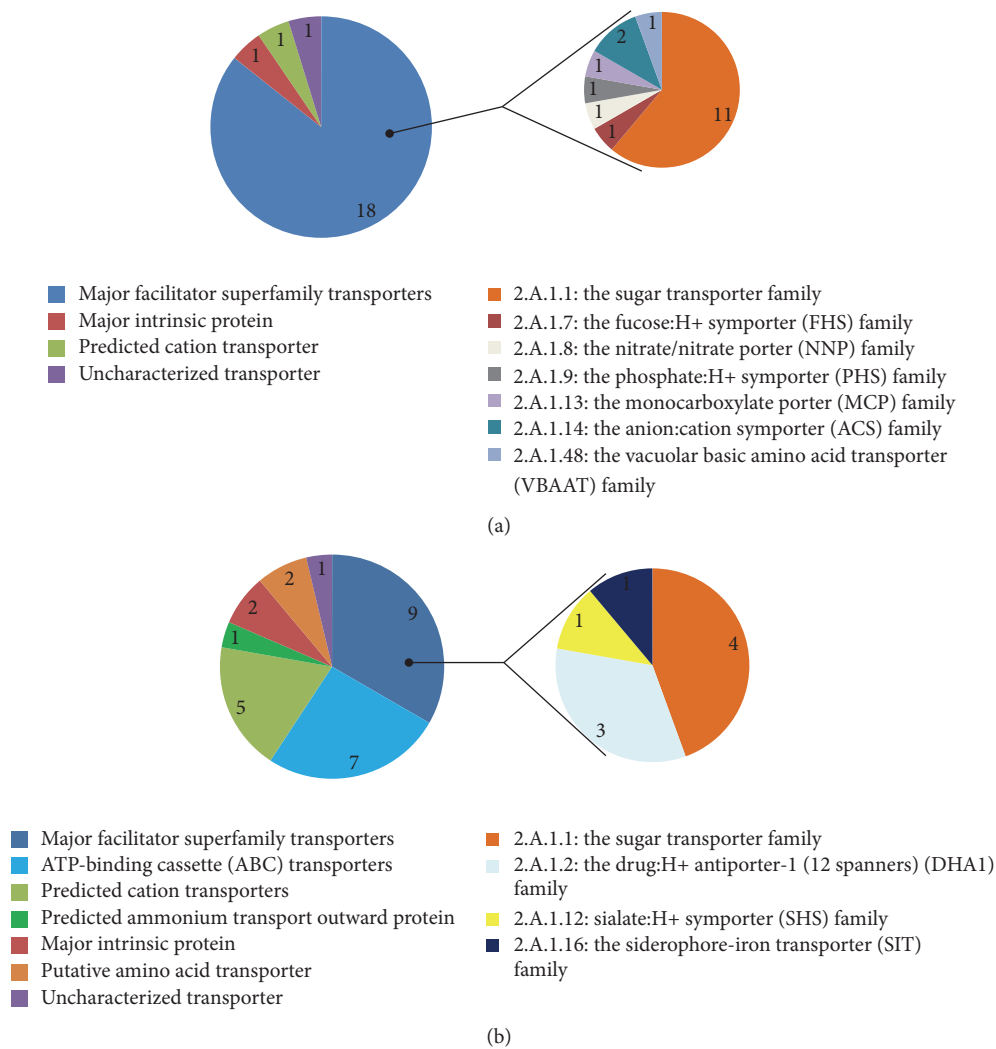


FIGURE 5: Distribution of putative transporters differentially expressed in $\Delta xyr1$ compared with Rut-C30 when cultured on lignocellulose. Distribution of putative transporters downregulated (a) and upregulated (b) in $\Delta xyr1$ compared with Rut-C30 when cultured under the lignocellulose culture condition. Details of major facilitator superfamily (MFS) transporter analysis are provided in the right panels of (a) and (b).

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 $\Delta xyr1$ 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 $\Delta xyr1$, is not known. Thus, two MFS transporter-encoding genes—the putative MFS glucose transporter *rco3* (TrireRUTC30: 136988) and the predicted MFS 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,

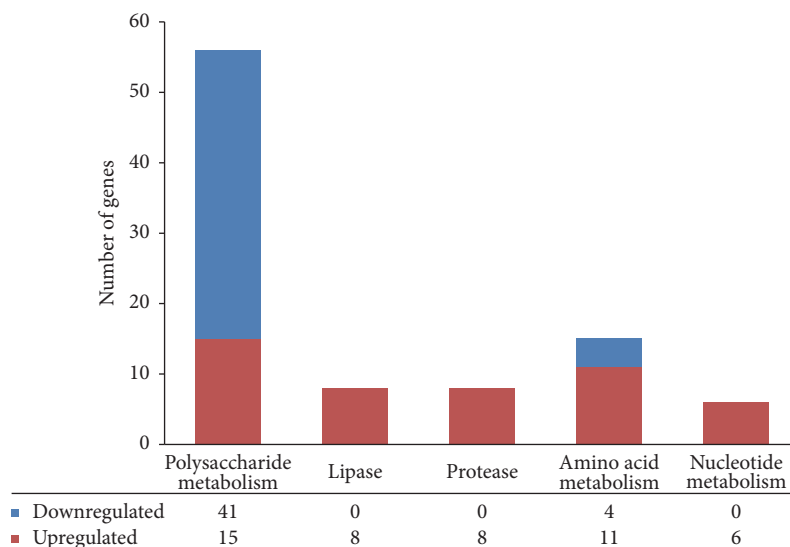


FIGURE 6: Distribution of differentially expressed genes involved in basal metabolism after *xyr1* deletion under lignocellulosic medium.

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 *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 on lignocellulose 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. 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, *endo T* (TrireRUTC30: 142298), was shown to be not involved in chitin degradation, but it has mannosyl-glycoprotein endo-*N*-acetyl- β -D-glucosaminidase activity [67]. It was reported to be responsible for *N*-deglycosylation of proteins expressed and secreted by *T. reesei* [68]. According to our RNA-seq data, the *endo T* in Rut-C30 was significantly upregulated under lignocellulose conditions (FPKM = 42.87) compared with glucose conditions (FPKM = 3.74). However, *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 *endo T* is very complex.

Foreman et al. [45] reported that *endo T* is not coregulated with the expression of cellulase genes in *T. reesei* Qm6a or RL-P37. Whether the increased expression of *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 *endo T* with Xyr1 (Figure 7(a)). In *T. reesei* RutC-30, the expression of *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 TrBgIS benefitted from its enzymatic activity and thermostability, compared with PpBgIS [69]. After deletion of *xyr1*, the upregulation of *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,

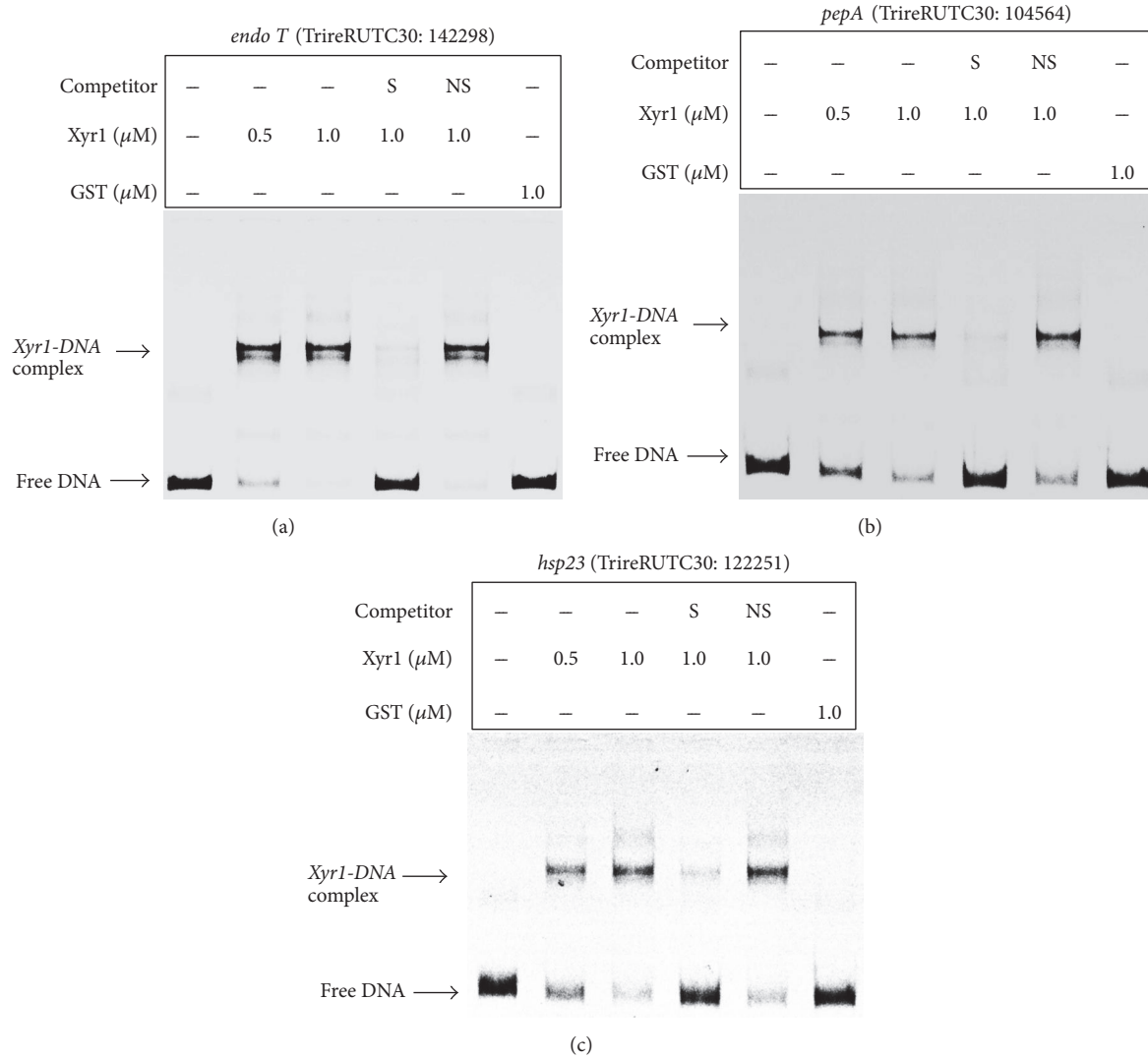


FIGURE 7: DNA binding of Xyr1 to the upstream regions of three genes upregulated after *xyr1* deletion. Xyr1 functionally binds to the promoter regions from the mannosyl-glycoprotein endo-*N*-acetyl- β -D-glucosaminidase, the putative acid aspergillopepsin I, and the possible heat shock protein Hsp23-encoding genes *endo T* (a), *pepA* (b), and *hsp23* (c), which were upregulated in $\Delta xyr1$ when cultured on the inducing medium. Strong gel shifts were observed after Xyr1 was added to the reactions. The protein-DNA complex increased with protein concentration. The amounts of purified Xyr1 (μM) used were as indicated and about 10 ng Cy5-labeled probe 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.

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 $\Delta xyr1$ (FPKM values ranged from 31.51 in Rut-C30 to 95.51 in $\Delta xyr1$). The α -1,2-mannosidase was reported to readily

convert $\text{Man}_8\text{GlcNAc}_2$, or a mixture of $\text{Man}_{(6-9)}\text{GlcNAc}_2$ oligosaccharides, to the respective Man_5 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 $\Delta xyr1$ 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 $\Delta xyr1$ (Table S5). In *Aspergillus nidulans*, an alcohol dehydrogenase (ADHII) 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 $\Delta xyr1$, under induced conditions compared with repressed conditions (Tables S7, S9).

The expression of TrireRUTC30: 26479 was also stimulated in $\Delta xyr1$ when transferred from the glucose to the lignocellulose condition (Table S9). In addition, TrireRUTC30: 87029, the *Podospora anserine* PaATG1 ortholog, was upregulated in $\Delta xyr1$ 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 developmental 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 $\Delta xyr1$ 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 $\Delta xyr1$ under a glucose condition, deletion of *xyr1* led to significant variation in a succinyl-CoA ligase gene (TrireRUTC30: 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 $\Delta xyr1$ 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 thermotolerance and result in higher biomass accumulation under thermal stress [77].

Under lignocellulose-inducing conditions, a gene homologous to *hsp23*, TrireRUTC30: 122251, was upregulated in $\Delta xyr1$ 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 consensus 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 $\Delta xyr1$, 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 (TrireRUTC30: 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 downregulated in $\Delta xyr1$ compared with the parent strain under a lignocellulose-inducing condition (Table S4).

Ace3 is a recently identified transcription activator of cellulase genes [78]. In $\Delta xyr1$, 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
<i>xyr1</i>	98788	43.73	42.93	No
<i>cre1-1</i>	23706	498.07	802.49	No
<i>ace1</i>	122363	337.92	200.20	No
<i>ace2</i>	32395	23.7	21.07	No
<i>ace3</i>	98455	81.56	137.33	No
<i>bglR</i>	91236	225.27	128.07	No
<i>hap2</i>	93466	39.57	21.40	No
<i>hap3</i>	24298	71.16	75.36	No
<i>clr-1</i>	68701	11.14	9.83	No
<i>clr-2</i>	76250	3.21	2.80	No
<i>nmrA</i>	121828	21.5	27.85	No
<i>areA</i>	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, $\Delta xyr1$ 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 *nmr*-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)

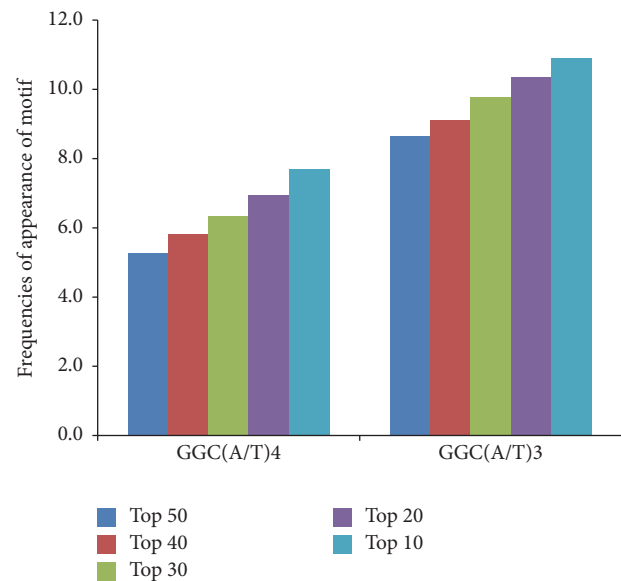


FIGURE 8: Frequencies of appearance of 5'-GGC(A/T)₃-3' and 5'-GGC(A/T)₄-3' motifs in the upstream regions of Xyr1-upregulated genes.

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 under the lignocellulolytic condition (Table S7). An average of 2.3 5'-GGC(A/T)₄-3' or 5.4 5'-GGC(A/T)₃-3' motifs was detected in the 1-kb 5'-upstream regions of 177 genes. An average of 5.3 5'-GGC(A/T)₄-3' motifs was detected in the top 50 Xyr1-upregulated genes, and the occurrence increased to 7.7 in the top 10 Xyr1-upregulated genes (Figure 8). Similarly, the

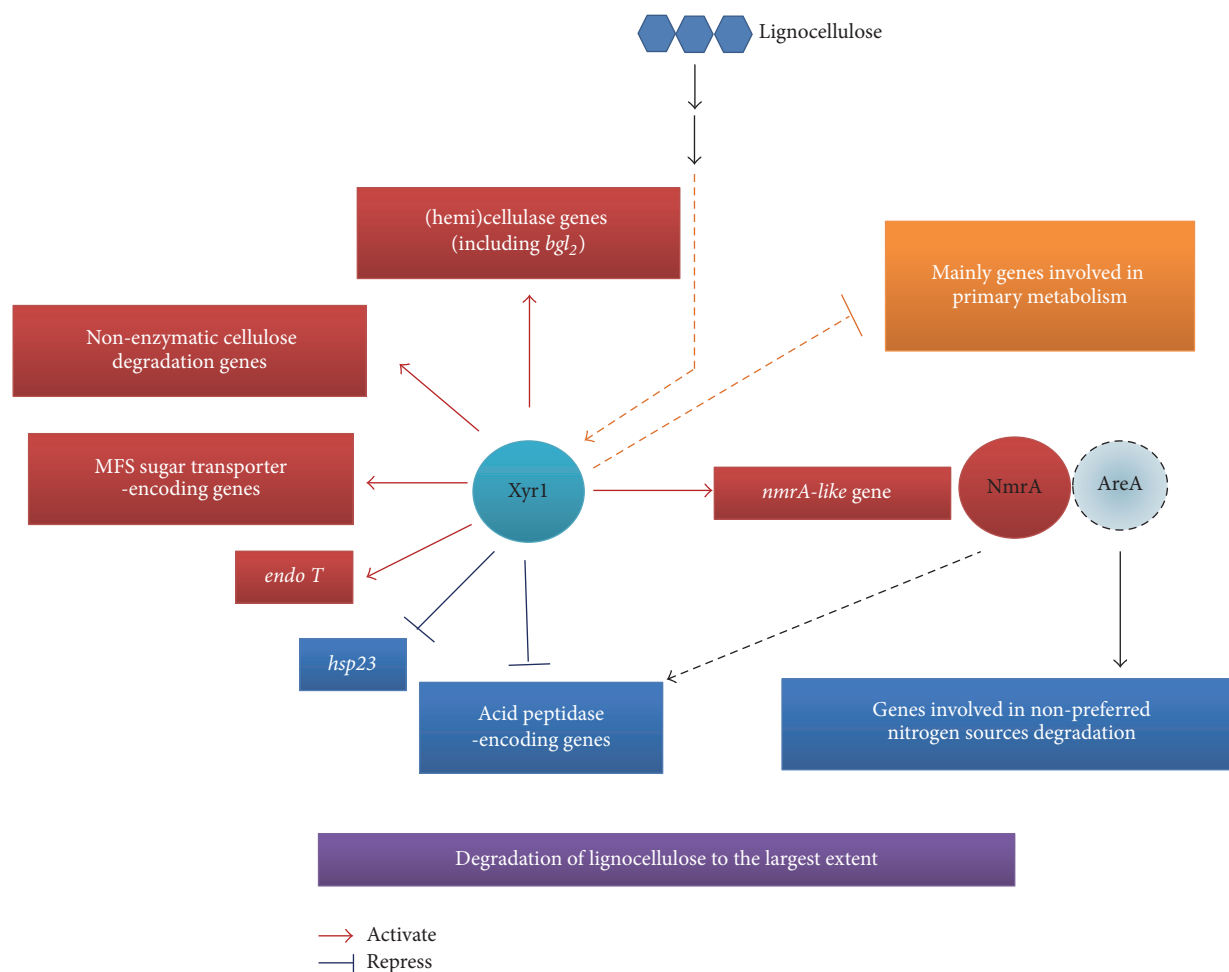


FIGURE 9: The predicted model of Xyr1-mediated gene regulation in *T. reesei* Rut-C30 under lignocellulose-inducing conditions. Xyr1 plays pleiotropic regulatory roles in the process of lignocellulose degradation and may be involved in primary metabolism. It is also implicated in interaction with the 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 *bgl2*, 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 (TriREUTC30: 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 *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. reesei*.

Abbreviations

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 $\Delta xyr1$ and *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.

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