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Transcriptional Profiling of the *Trichoderma reesei* Recombinant Strain HJ48 by RNA-Seq

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The ethanol production of *Trichoderma reesei* was improved by genome shuffling in our previous work. Using RNA-Seq, the transcriptomes of *T. reesei* wild-type CICC40360 and recombinant strain HJ48 were compared under fermentation conditions. Based on this analysis, we defined a set of *T. reesei* genes involved in ethanol production. Further expression analysis identified a series of glycolysis enzymes, which are upregulated in the recombinant strain HJ48 under fermentation conditions. The differentially expressed genes were further validated by qPCR. The present study will be helpful for future studies on ethanol fermentation as well as the roles of the involved genes. This research reveals several major differences in metabolic pathways between recombinant strain HJ48 and wild-type CICC40360, which relates to the higher ethanol production on the former, and their further research could promote the development of techniques for increasing ethanol production.

Keywords: *Trichoderma reesei*, transcriptome, RNA-Seq, qPCR

Introduction

One potential strategy to decrease greenhouse gas emissions and, thus, global warming, is through bioethanol production from the substrate lignocellulose. However, this manufacturing strategy is predominantly limited by its high cost, which is due to the high expense of pretreatment and cellulase enzymes. An important method of reducing this cost is through consolidated bioprocessing (CBP), but the major bottleneck of which is the development of CBP organisms capable of facilitating this process [28].

Traditionally, there are two primary developmental paths followed when producing CBP strains. The first consists of taking a category I cellulose-producing CBP organism and giving it ethanol-producing capabilities (*e.g.*, *Thermoanaerobacterium saccharolyticum*). The second is essentially a reverse of the first, where an ethanol-producing category II CBP organism is engineered to be cellulolytic (*e.g.*, *Pachysolen tannophilus*) [3, 7, 10]. Thus far, a number of studies have been published that used genetic engineering to create yeast strains expressing cellulase on the cell surface. However, the expression levels of cellulase in yeast strains have typically been low, and, therefore, the generation of category II CBP organisms for biorefinery is primarily limited by the inability to produce adequate quantities of the active form of cellulase. Thus, category I CBP organisms are the more attractive of the two strategies when optimizing bioethanol production, and these cellulolytic bacteria offer a number of other advantages. First, they are not strict anaerobes, and thus can be directly deposited onto cellulose-containing biomasses. Filamentous fungi have been identified that are capable of breaking down lignin, an inhibiter of lignocellulose saccharification, while others have been discovered that are able to make ethanol from cellulose (*e.g.*, *Aspergillus*, *Rhizopus*, *Monilia*, *Neurospora*, *Fusarium*, and *Trichoderma*) [9, 19, 24, 25].

Trichoderma reesei is an organism often used by industry as a source of cellulases and hemicellulases, which are enzymes that break down cellulose and hemicellulose, respectively. Of the genetic studies published focusing on this organism, most have characterized the molecular mechanisms behind its production of cellulase [8, 20, 23]. One study measured and compared the *T. reesei* transcriptome during growth on different carbon sources, such as wheat straw and lactose, and found differences between these conditions that may be related to enzyme expression. Further investigation could reveal ways to amplify enzyme production when *T. reesei* is grown on lactose [5]. Further work on *T. reesei* was performed by Häkkinen *et al.*, who profiled transcription of the organism following induction of cellulose and hemicellulose using lignocellulose-derived compounds, allowing them to isolate potentially novel regulators of these genes [11]. Another study on *T. reesei* used RNA-Seq to quantify the global induction of genes upon exposure to wheat straw, and compared this response to wheat straw with the response by another organism, *A. niger* [21]. A similar expression pattern of the enzymes used to break down solid lignocelluose was found between the two organisms, suggesting conservation of this function in both saprobic fungi [14]. One study suggests *T. reesei* as a good candidate for CBP; however, there needs to be optimization to significantly increase its ethanol production [3, 26]. To accomplish this, we used a genome shuffling technique using *S. cerevisiae* and *T. reesei* that consisted of recursive direct genome transformation rather than recursive protoplast fusion to rapidly create a recombinant strain [12]. However, there are some obstacles to overcome with HJ48 before it would be useful for CBP, including its ethanol yield, rate of production, and tolerance.

Cellulosic sugars are converted into ethanol via a number of metabolic pathways, including fermentation, glycolysis, and the pentose phosphate pathway, the genes for which are all encoded by *T. reesei.* However, expression of the genes involved in the fermentation of ethanol from glucose has not been reported on in this organism, and deepening our understanding of the genetics of *T. reesei* is necessary to reveal its full biotechnological potential. RNA-Seq was used to quantify the expression of genes and identify those differentially expressed between *T. reesei* CICC40360 and HJ48, with a focus on ethanol production [12]. The results increase our knowledge on the reactions involved in ethanol fermentation at the transcriptional level, and assist in other studies characterizing the biotechnological potential of *T. reesei.*

Materials and Methods

Strains and Fermentation

T. reesei CICC40360 and its recombinant strain HJ48 were used in this research. The growth medium (GM) contained 4 g glucose, 5 g yeast extract, and 3 g potato extract per liter. The fermentation medium (FM) contained 50 g glucose, 10 g yeast extract, 10 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, and 0.5 g $MgSO_4{\cdot}7H_2O$ per liter.

The fermentation of *T. reesei* was carried out as previously described [12]. Fungal mycelia were grown on a PDA agar plate for 7 days and then three loops of the mycelium mat were inoculated into 500 ml of GM in a 1 L Erlenmeyer flask. After incubation for 4 days at 180 rpm at 30°C, the mycelium was harvested (20 ± 2 g, wet weight) and transferred aseptically to a 100 ml Erlenmeyer flask containing 20 ml of FM. FM without sugar was used as the control. In aerobic conditions, the Erlenmeyer flasks were covered with cotton and shaken at 130 rpm.

RNA Extraction

Total RNAs were extracted using TRIzol reagent, and then purified using the NanoPhotometer spectrophotometer. RNA Qubit RNA Assay Kit (Life Technologies, USA) and Nano 6000 Assay Kit (Agilent Technologies, USA), respectively, were used to check the RNA concentration and integrity.

Sample Preparation for Sequencing

Four micrograms of RNA per sample was used for the RNA sample preparations. The NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) was used to create sequencing libraries, and index codes were added to attribute sequences to each sample. Poly-T oligo-attached magnetic beads were used to purify mRNA from total RNA. Fragmentation was performed with divalent cations under elevated temperature in NEB Next First Strand Synthesis Reaction Buffer (5×). cDNA synthesis was performed according to a previous report [29].

Clustering and Sequencing

The clustering of the index-coded samples was used on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia). The library preparations were sequenced on an Illumina Hiseq 2500 platform, and 100 bp paired-end reads were generated.

Quality Control

Trimmed data of fastq format were performed through in-house perl scripts. Trimmed data were performed by removing adapter, reads containing ploy-N, and low-quality reads from the trimmed data. Meanwhile, the Q20, Q30, and GC content of the trimmed data were calculated. The further research was based on the trimmed data.

Reads Mapping to the Reference Genome

The *T. reesei* genome and gene model annotation files were

downloaded from the Internet directly (http://genome.jgi-psf.org/Trire2/Trire2.home.html) [16]. Bowtie v2.0.6 was used to build the index of the reference genome and paired-end clean reads were aligned to the reference genome by TopHat v2.0.9 [13].

Quantification of Gene Expression Level

Count of the read numbers mapped to each gene was implemented by the HTSeq v0.6.1 [2]. RPKM of each gene was calculated based on the length of the gene and read counts mapped to this gene [17].

Differential Gene Expression Analysis

Before differential gene expression analysis, for each sequenced library, the edgeR program package via one scaling normalized factor was used to adjusted the read counts [22]. The DEGSeq R package (1.12.0) was used to analyze the differentail expression of two conditions, a qvalue (corrected *p*-value) <5 and log2 (fold-change) >1. (The qvalue is an estimation of the false discovery rate (FDR) optimized version of the *p*-value.) [27]. The *p*-value was adjusted by the Benjamini method [4].

GO and KEGG Enrichment Analysis of Differentially Expressed Genes

The GOseq R package was used to test gene ontology (GO) enrichment analysis of differentially expressed genes, in which gene length bias was corrected. GO terms with corrected *p*-value less than 0.05 were considered significantly enriched by differential expressed genes [1].

The statistical enrichment of differential expression genes in KEGG pathways was implemented by KOBAS software [15].

qPCR

qPCR was implemented by a CFX 96 real-time PCR detection system with reagents from TOYOBO. The PCR mixture (20 μl) with three replicates was implemented according to the manufacturer's instruction. The method of $2^{-\Delta\Delta Ct}$, with expression in strain CICC40360 as the control and expression of actin as an internal standard, was used to test the relative transcript level of each gene. Primers were showed in Table 3.

Results and Discussion

The Fermentation Performance of *T. reesei*

The concentration of ethanol was measured to observe the fermentation performance of *T. reesei* during the fermentation period using glucose as a carbon source. When parent strain CICC40360 and recombinant strain HJ48 were grown on aerobic conditions using glucose as carbon source, after 96 h of cultivation, the sugar was completely consumed by both fungi. The maximum ethanol production was observed after 96 h, after which the ethanol concentration was decreased owing to the consumption of glucose (Fig. 1). A 48 h adaptation period was included in this 96 h fermentation phase. The increased ethanol production showed the improved ability of *T. reesei* to ferment glucose to ethanol. In order to compare the expression of genes that were induced in *T. reesei* between CICC40360 and HJ48 during glucose conversion to ethanol, total RNA from the mycelia at 96 h of fermentation was extracted.

Summary of RNA-Seq Data Sets

High-throughput profiling of transcripts under different conditions is an incredibly useful method for studying a

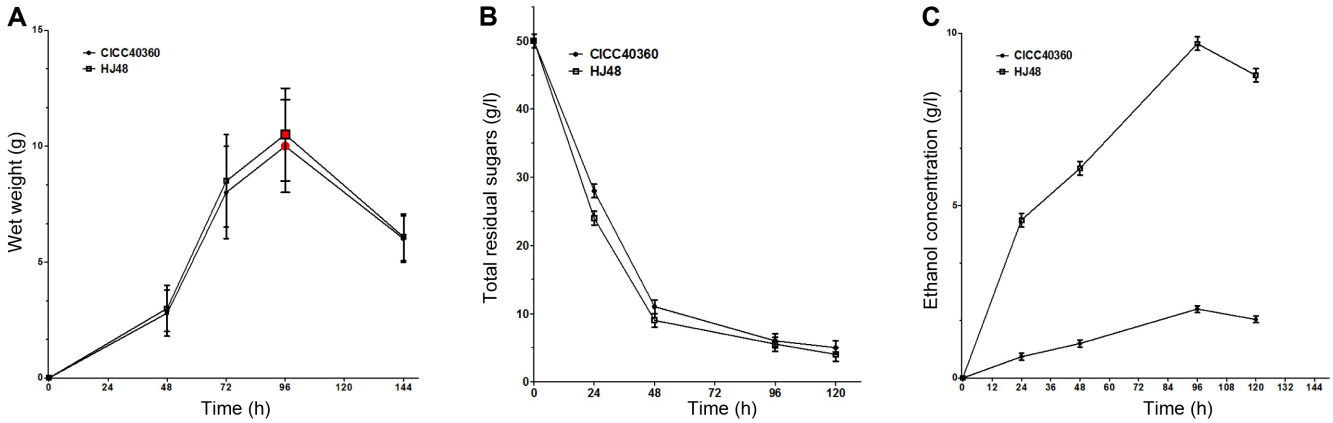

Fig. 1. Fermentation performances of *Trichoderma reesei* strain CICC40360 and recombinant strain HJ48.
The mycelia that were pre-cultured on GM for 4 days (red point) were washed and transferred aseptically to FM (**A**). The Erlenmeyer flasks were shaken at 130 rpm and 30°C. Samples were withdrawn periodically to determine the concentration of sugar and ethanol. Time courses of glucose consumption (**A**) and ethanol production (**B**) by HJ48 and CICC40360. The values are means of triplicate experiments. Error bars indicate standard deviations.

large number of genes, and it was used in this study to compare the gene expression of strains HJ48 and CICC40360 under fermentation inducing conditions. First, total RNA was purified from four samples, tested for quality, and then an RNA sample from each strain was prepared and sent for Illumina sequencing. The resulting sequence data were deposited in NCBI's Sequence Read Archive public archive database with the accession number SRR: 1997914.

The resulting Illumina reads were mapped against a published *T. reesei* genome, and the overall transcriptional activity was calculated for each gene by calculating the number of reads per kilobase of exon model per million mapped reads (RPKM). A summary of all the *T. reesei* genes with detectable levels of expression is presented in Table S1. Differential expression analysis was conducted using DESeq with a q value <0.005 and $\log_2$(fold-change) >1 [4]. Overall, 3,222 genes were expressed at different levels in HJ48 and CICC40360, where 2,106 genes were upregulated and 1,116 genes were downregulated. The comparison between the gene expression levels of the two samples is shown in Fig. 2.

These genes differentially expressed between HJ48 and CICC40360 were further analyzed using GOSeq, which assigns GO in order to identify putative functions for these genes, as shown in Fig. 3 [30]. This method uses gene sequence homology to functionally categorize genes of interest based on common biological properties, such as biological processes, cellular components, and molecular functions. The genes that were categorized as being part of biological processes were split into four subcategories, where 592 were involved in single-organism metabolic processes [GO: 0044710], 232 in small molecule metabolic processes [GO: 0044281], and 136 genes in oxoacid metabolic

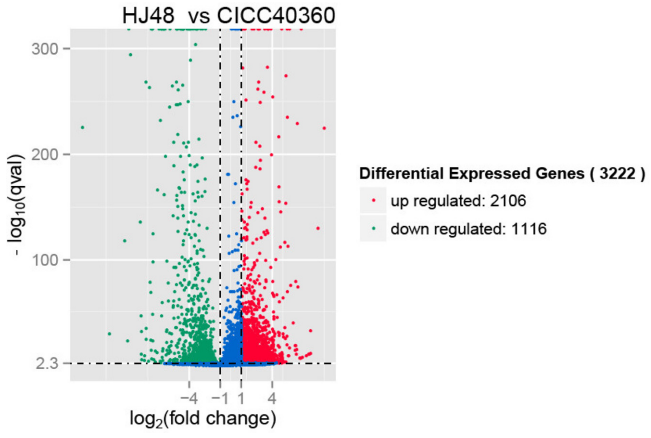

Fig. 2. Overview of the differentially expressed genes between CICC40360 and HJ48.

[GO: 0043436] and organic acid metabolic processes [GO: 0006082]. Twenty-six genes are considered cellular components, which include parts of the endoplasmic reticulum (ER) membrane [GO: 0005789] and nuclear outer membrane-ER membrane network [GO: 0042175]. Another 21 genes were assigned into the molecular function category and split into two main subcategories, where 13 genes had oxidoreductase activity and acted on single donors with incorporated molecular oxygen [GO: 0016703] and 8 genes had glutamate synthase activity [GO: 0015930]. Interestingly, all of the genes that fell into DNA polymerase III complex and DNA polymerase complex related categories were upregulated in HJ48 compared with the control strain.

In order to categorize the genes differentially expressed between the recombinant strain and parental strain into functional pathways, the genes were classified based on KEGG enrichment using FDR ≤0.05. The genes with different expression levels between HJ48 and CICC40360 were predominantly functionally categorized into 20 pathways, including protein processing in the ER [PATHWAY: tre04141], lysine degradation [PATHWAY: tre00310], tryptophan metabolism [PATHWAY: tre00380], glycerophospholipid metabolism [PATHWAY:tre00564], glycerolipid metabolism [PATHWAY: tre00561], pentose phosphate pathway [PATHWAY: tre00030], glycolysis/gluconeogenesis [PATHWAY: tre00010], citrate cycle (TCA cycle) [PATHWAY: tre00020], DNA replication [PATHWAY: tre03030], and base excision repair [PATHWAY: tre03410] (in Table S2). Statistical analysis revealed that the differentially expressed genes were enriched for those involved in protein processing in the ER [PATHWAY: tre04141]. Although the differentially expressed genes were enriched for the pentose phosphate pathway [PATHWAY: tre00030], glycolysis/gluconeogenesis [PATHWAY: tre00010], TCA cycle [PATHWAY: tre00020], DNA replication [PATHWAY: tre03030], and base excision repair [PATHWAY: tre03410], this enrichment was not statistically significant (FDR ≤0.05) (Table S2).

Analysis of Genes Involved in Glucose Transport

Transport of glucose across the plasma membrane has been suggested to be an essential first step of glucose metabolism. Several of the genes found to be differentially expressed between HJ48 and the parental strain may be involved in glucose transport based on their homology to genes in other organisms as determined by Swiss Prot comparisons (Table 1). The gene TRIREDRAFT_54005 is homologous to a putative low glucose sensor in *S. cerevisiae*, which is suggestive of it performing a similar function in

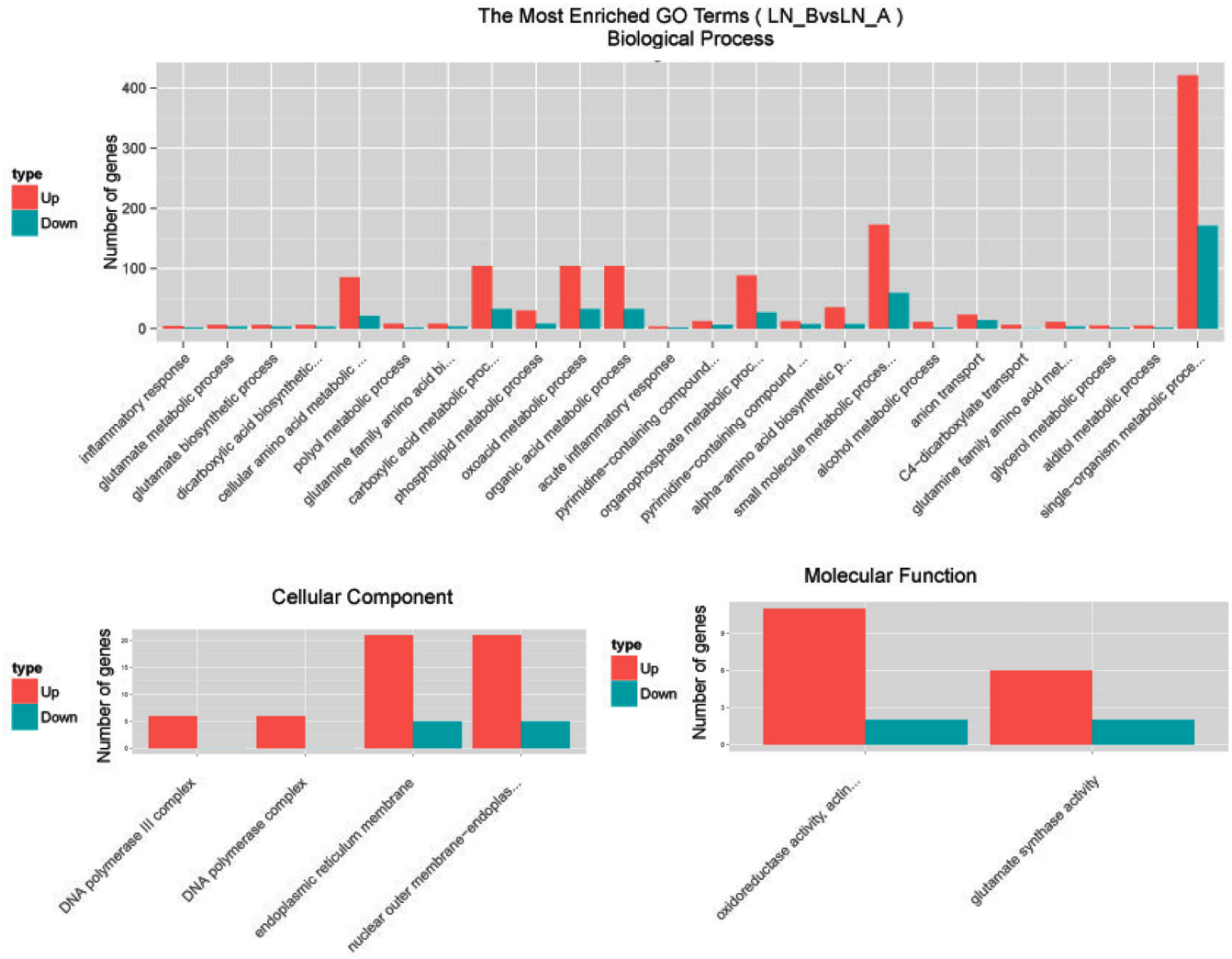

Fig. 3. Histogram of gene ontology classification.
Gene ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOseq R package. The results are summarized in three main categories: biological process, cellular component, and molecular function.

T. reesei. The *S. cerevisiae* homolog of TRIREDRAFT_54005, SNF3, encodes for a glucose receptor that binds extracellular glucose and generates an intracellular signal for induction of hexose transporter (HXT) gene family expression. This suggests that HJ48 is more sensitive to glucose than CICC40360. Another gene, TRIREDRAFT_60086, has a homolog in *Kluyveromyces lactis* (HGT1) involved in high-affinity glucose transport, whereas TRIREDRAFT_62502 has homologs in *S. cerevisiae* that serve as sugar transporters.

Furthermore, glucose transport across the plasma membrane into cells is regulated by permeases that belong to the major facilitator superfamily (MFS) [18]. Several MFS genes were differentially expressed by HJ48 and CICC40360. This suggests differences in glucose transport between the two strains, and, therefore, HJ48 may be better able to import sugar into the cell than CICC40360, allowing it to outcompete it. Overall, the results based on high sequence homology suggest that sugar transporters in *T. reesei* and yeast strains may behave similarly, could play comparable roles in fermentation of wine, and are an important direction for further research.

Analysis of Genes Involved in the Central Carbon Metabolism

In this study, we characterized and compared the expression of genes involved in glycolysis and alcoholic fermentation by measuring transcript levels in two *T. reesei* strains (Fig. 4). Overall, there was little change in the

Table 1. The upregulated expression of predicted sugar transporter genes in HJ48.

Gene_id	qvalue	BLAST Swiss Prot
TRIREDRAFT_54005	1.05E-07	Yeast High-affinity glucose transporter
TRIREDRAFT_60086	1.41E-17	High-affinity glucose transporter
TRIREDRAFT_62502	3.97E-05	Yeast Sugar transporter STL1
TRIREDRAFT_110843	5.55E-06	Uncharacterized MFS-type transporter
TRIREDRAFT_123702	9.20E-39	Uncharacterized MFS-type transporter PB1E7.08c
TRIREDRAFT_54036	4.29E-11	Uncharacterized MFS-type transporter C1683.03c
TRIREDRAFT_65744	0.004134	Uncharacterized MFS-type transporter C1399.02
TRIREDRAFT_79116	7.14E-35	Uncharacterized MFS-type transporter PB1E7.08c

quantities of glycolytic pathway enzyme transcripts in the presence of glucose, which is consistent with previously published reports [6]. Importantly, genes in the upstream part of the glycolytic pathway were transcribed at significantly higher levels in HJ48 than CICC40360 (Table 2). For example, there was a higher abundance of transcripts in HJ48 of the two isoforms of the hexokinase (TRIREDRAFT_73665 and TRIREDRAFT_80231) that catalyzes the first irreversible reaction in glycolysis. In addition, glucose-6-phosphate (TRIREDRAFT_5776) and glucose-6-phosphate 1-epimerase (TRIREDRAFT_22415), which convert glucose-6-phosphate into fructose-6-phosphate, as well as phosphoglucomutase (TRIREDRAFT_21836) and aldose-1-epimerase (TRIREDRAFT_120784) were expressed at higher levels in HJ48 than CICC40360. On the other hand, fructose-bisphosphatase, (TRIREDRAFT_65508), which converts fructose-1,6-bisphosphate into fructose-6-phosphate, was downregulated in HJ48, and, 6-phosphogluconolactonase (TRIREDRAFT_73903), an enzyme important for early steps of the pentose phosphate pathway, was expressed at relatively low levels in HJ48. This suggests that the main carbon flow for the recombinant strain HJ48 may be through glycolysis to produce pyruvate.

Under conditions conducive to fermentation, few differences were found between the two strains when measuring transcripts of TCA cycle genes, including in citrate synthase, aconitate hydratase, isocitrate dehydrogenase, and fumarate hydratase, with the notable exception of two

Table 2. Up/downregulated expression of genes involved in the metabolic pathways controlling the production of ethanol.

Gene_id	log2 Fold_change	qvalue	Function
TRIREDRAFT_120784	1.6055	0.00084503	Aldose 1-epimerase
TRIREDRAFT_73665	2.0816	1.06E-37	Hexokinase
TRIREDRAFT_80231	1.5217	8.73E-11	Glucokinase
TRIREDRAFT_22415	1.8222	5.54E-11	Glucose-6-phosphate 1-epimerase
TRIREDRAFT_21836	1.0293	1.23E-14	Phosphoglucomutase
TRIREDRAFT_5776	1.601	2.68E-51	Glucose-6-phosphate isomerase
TRIREDRAFT_65508	-2.373	1.52E-18	Fructose-1,6-bisphosphatase
TRIREDRAFT_122736	1.4015	0.003819	Putative fructose-bisphosphate aldolase
TRIREDRAFT_121789	1.1647	3.66E-13	Triosephosphate isomerase
TRIREDRAFT_68606	2.6744	9.75E-06	Putative triosephosphate isomerase
TRIREDRAFT_121240	1.9113	5.78E-07	Sedoheptulose 1,7-bisphosphatase
TRIREDRAFT_77656	-1.9555	0.0002992	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase
TRIREDRAFT_22633	1.9579	1.01E-43	Glutathione dehydrogenase
TRIREDRAFT_78683	-2.0184	8.58E-15	Aldehyde dehydrogenase
TRIREDRAFT_124115	-1.5747	1.69E-14	Phosphoenolpyruvate carboxykinase
TRIREDRAFT_50531	-1.5694	5.21E-07	2-Oxoglutarate dehydrogenase
TRIREDRAFT_80881	-1.6884	4.50E-05	Probable succinyl-CoA ligase
TRIREDRAFT_73903	-3.8757	4.24E-28	6-Phosphogluconolactonase

genes that were significantly downregulated in HJ48 (Table 2). These results may assist in the elucidation of the role of the pyruvate pathway during fermentation. Previously published studies have shown that glucose is not a negative regulator of tricarboxylic acid cycle genes in *T. reesei* [28]. Rather, oxidation of pyruvate is accelerated by increasing the expression of TCA cycle enzymes when there are high intracellular glucose levels. However, transcription of these genes was lower in HJ48 than CICC40360, suggesting glucose is strongly inhibiting TCA cycle gene expression and channeling pyruvate into acetaldehyde in HJ48 in a manner similar to that of *S. cerevisiae*.

Pyruvate is converted into acetaldehyde by pyruvate decarboxylase (TRIREDRAFT_59267), a gene upregulated in HJ48, via non-oxidative decarboxylation in *T. reesei*. Moreover, the pyruvate dehydrogenase complex E1-α subunit (TRIREDRAFT_122987), a gene needed for respiratory catalysis of pyruvate into acetyl-CoA, was downregulated in HJ48. A striking difference between HJ48 and its parental strain concerns the fate of acetaldehyde as inferred from transcript levels of alcohol dehydrogenase genes. In this study, we identified two alcohol dehydrogenase paralogs in *T. reesei*, and the low expression observed of these alcohol dehydrogenases (TRIREDRAFT_56839) suggests that the production of acetaldehyde from ethanol is inhibited in HJ48. However, there were high expression levels in HJ48 of the alcohol dehydrogenase (TRIREDRAFT_22633) catalyzing the reverse reaction that creates ethanol from acetaldehyde in a reductive process. This results in an increase in ethanol production from acetaldehyde under these conditions and clearly demonstrates that overexpression of alcohol dehydrogenase is necessary to make *T. reesei* an effective ethanol producer under both anaerobic and aerobic conditions.

Phosphoenolpyruvate carboxykinase catalyzes phosphoenolpyruvate production from oxaloacetate, regulates ethanol production, and becomes activated when there is

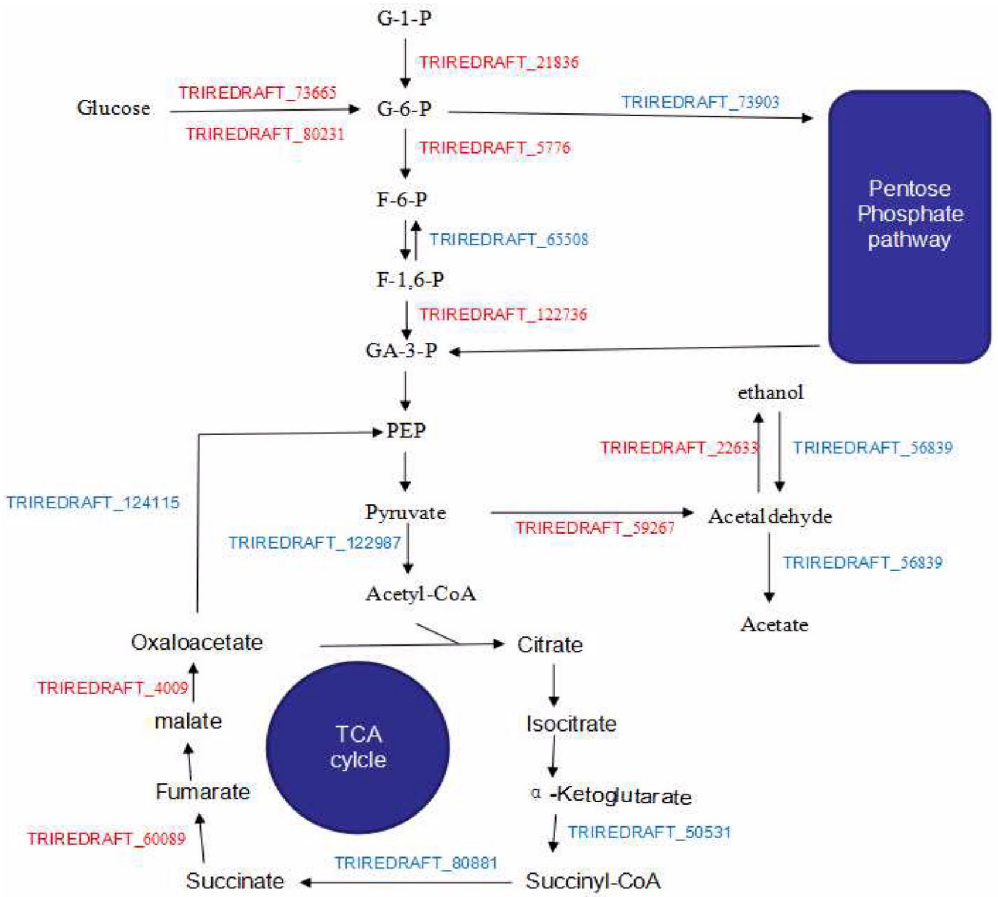

Fig. 4. Differential expression of genes involved in the central carbon metabolism.
Red and blue represent those genes whose expression was upregulated and downregulated, respectively (TCA cycle, citrate cycle).

an increased amount of acetate. However, the expression of this gene was significantly decreased in HJ48 compared with the parental strain, indicating that the conversion of acetaldehyde into ethanol rather than acetate is the main metabolic pathway for HJ48.

The expression of the genes of interest involved in ethanol-producing metabolic pathways were confirmed by real-time PCR (qPCR) (Fig. 5), and the results were in agreement with those from RNA-Seq.

In this part, the expression of genes involved in glycolysis and alcohol fermentation during glucose fermentation was compared between the recombinant and parental strains. It was found that there was high expression of most of the glycolytic genes in HJ48 compared with the parental strain, but this expression was not significantly affected by high glucose levels during fermentation. For example, 5 out of 7 genes in the upstream portion of the glycolytic pathway had slightly upregulated expression levels during fermentation. The expression levels of several genes of interest, chosen for their role in catalyzing irreversible reactions during glycolysis or alcohol fermentation, were measured by qPCR (Fig. 5), and it was found the qPCR results paralleled the RNA-Seq data. Although transcription of *T. reesei*'s central metabolic pathways has been shown to not be the major point of control [6], entry into these metabolic pathways is likely an important area of control and could be regulated at both the metabolic and transcriptional levels. This multilevel regulation would allow yeast cells to have tight control on metabolic pathways and, thus, maximize metabolic efficiency under a number of conditions. A strong correlation between rates of metabolism and gene expression was also identified for the initial steps in metabolic pathways in *Corynebacterium glutamicum* [14], suggesting that this type of regulation mechanism is likely common to a wide range of microorganisms. Further work remains to be done to fully characterize the regulation of central metabolic pathways, including the roles of post-transcriptional mechanisms and modifications, and signaling by intracellular concentrations of substrates, products, and effectors.

Table 3. Quantitative PCR primers of the candidate genes.

Name of gene	Primer sequences (5′-3′)
73665-qF	CTCTTGGCTTCACCTTCTCCTACC
73665-qR	AATGTGCCTGTCGTGTCGTTGAT
80231-qF	GGACAAGATTCGCATCGGCATCG
80231-qR	TCGCAGGTCGGCAAGGAAGT
5776-qF	GCCTACTACGAGCACCTGACCTT
5776-qR	CAATGAGACCGCCAGTGGAAGC
21836-qF	GTCTATCTGCGGTGAGGAGAGTT
21836-qR	CGTAGTCGTATCTGGTGAAGAATGTG
122736-qF	GCTTGATGACGACGATGGAGGAG
122736-qR	TGAGGACATTGCTTGCACTGGTAAT
59267-qF	AGCCGTGGAGGAACACTCTG
59267-qR	CTTCTTGAGCAACCTTGGAGCAT
22633-qF	GTTCATCACGCACCGCAAGA
22633-qR	TTACAACTTCCGCATGTCAACAAC
78683-qF	TCGCCGCCATTGCCAAGTT
78683-qR	GCAGCAGGTTGTAGCAGTTGAC
122987-qF	CGCAAGAACGCCAGTGTGAT
122987-qR	TCGCTTGTAGTAGTCGGTCAGG
124115-qF	CCAGACCATGTTCCACTTCATCTCC
124115-qR	GCCTTGTGCTGCTCAATCTTGTC
73903-qF	CCGCCTTGCTGGTTGCTTCAT
73903-qR	CGTAGAGATTGGGCTGTGTCTTGG
56839-qF	AAGATGCTCAACCTGTGTCTGATTGG
56839-qR	ACTGAATCGCCTCCTCGCTGTC

Analysis of Genes Involved in Protein Synthesis

Manufacturing of heterologous or extracellular fungal

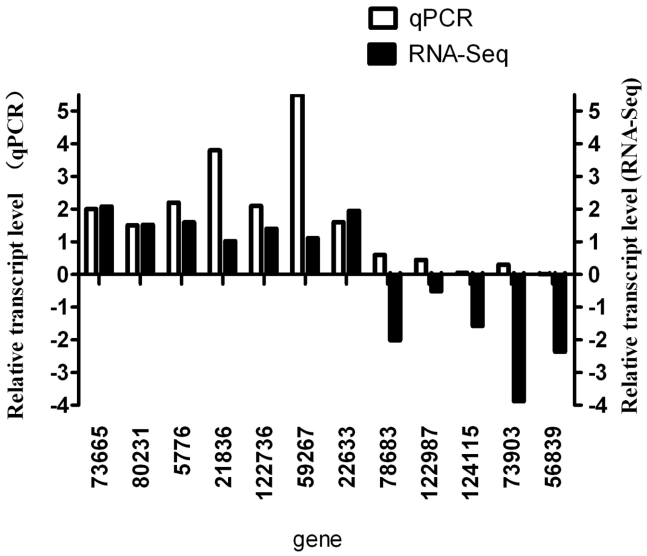

Fig. 5. Quantitative PCR analysis of the candidate genes.
The expression levels of candidate genes were monitored by quantiative real time-PCR. The qPCR results and RNA-Seq expression were in agreement. The experiment used actin as the internal standard. The values shown are the means of three independent experiments (73665 and 80231: HK; 5776: PGI; 21836: PGM; 122736: FBA; 59267: PDC; 22633: ADH1; 78683: ALD; 122987: PDA; 124115: PCK; 73903: PGLS; 56839: ADH2).

proteins on a large scale by *T. reesei* creates a need by the organism to optimize the process and increase the efficiency of the synthesized protein modification, transport, and quality control [8]. In this study, genes associated with these types of processes, such as protein glycosylation and ER transport, were upregulated in HJ48 (Table S3). Fifty percent of proteins are approximated to be glycoproteins, and, therefore, a high rate of transcription of protein glycosylation genes is necessary for increasing protein modification in HJ48. Furthermore, the need to manufacture large quantities of proteins in turn creates a need to enhance the efficiency of protein transport. Interestingly, ER degradation-associated genes were downregulated in HJ48, whereas ribosomes and ribosome biogenesis were more active in HJ48 than CICC40360. These results show that, in contrast to the parental strain CICC40360, HJ48 was significantly more efficient at protein modification and secretion.

There were higher rates of transcription in HJ48 of genes associated with MCM complex (helicase) activity, which play important roles in DNA replication and cell division. Along these lines, our study found that DNA replication and base excision repair occurred at much higher levels in HJ48 than CICC40360 (Table S3), which may be related to the fast growth and hyphal differentiation with asexual development of HJ48.

This study provides the first analysis of the *T. reesei* transcriptome and compares the recombinant strain HJ48 to its parental strain CICC40360 under conditions conducive to fermentation. Interestingly, dramatic differences were found between the transcription profiles of these two strains, as well as an amplified response involving transport of sugars, manufacturing of ethanol, glycosylation of proteins, and transport through the ER. This study increases our knowledge on the mechanism behind the conversion of glucose into ethanol by filamentous fungi. Furthermore, the transcriptome data can serve as an important resource when utilizing metabolic engineering of filamentous fungi for the generation of biofuels.

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