Regular Articles

# Defining the genome-wide role of CRE1 during carbon catabolite repression in *Trichoderma reesei* using RNA-Seq analysis

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A R T I C L E   I N F O

A B S T R A C T

The ascomycete *Trichoderma reesei* is one of the most well-studied cellulolytic fungi and is widely used by the biotechnology industry in the production of second generation bioethanol. The carbon catabolite repression (CCR) mechanism adopted by *T. reesei* is mediated by the transcription factor CRE1. CCR represses genes related to cellulase production when a carbon source is readily available in the medium. Using RNA sequencing, we investigated CCR during the synthesis of cellulases, comparing the *T. reesei* Δ*cre1* mutant strain with its parental strain, QM9414. Of 9129 genes in the *T. reesei* genome, 268 genes were upregulated and 85 were downregulated in the presence of cellulose (Avicel). In addition, 251 genes were upregulated and 230 were downregulated in the presence of a high concentration of glucose. Genes encoding cellulolytic enzymes and transcription factors and genes related to the transport of nutrients and oxidative metabolism were also targets of CCR, mediated by CRE1 in a carbon source-dependent manner. Our results also suggested that CRE1 regulates the expression of genes related to the use of copper and iron as final electron acceptors or as cofactors of enzymes that participate in biomass degradation. As a result, the final effect of CRE1-mediated transcriptional regulation is to modulate the access of cellulolytic enzymes to cellulose polymers or blocks the entry of cellulase inducers into the cell, depending on the glucose content in the medium. These results will contribute to a better understanding of the mechanism of carbon catabolite repression in *T. reesei*, thereby enhancing its application in several biotechnology fields.

## 1. Introduction

*Trichoderma reesei* (*Hypocrea jecorina*) is a saprophyte fungus widely used in the biotechnology industry because of its large cellulolytic potential (Schuster and Schmoll, 2010). To ensure its survival in different habitats, *T. reesei* must detect cellulose in its environment, produce different cellulases to degrade the insoluble substrate, carry the soluble products across the cytoplasmic membrane, and assimilate the sugars. In addition, *T. reesei* must respond to changes in the nutritional composition of the environment in order to compete with other microorganisms (Seiboth et al., 2011). An important mechanism for controlling metabolic processes in prokaryotes and eukaryotic microorganisms is carbon catabolite repression (CCR), in which the expression of genes necessary for the use of alternative carbon sources is repressed in the presence of easily metabolized carbon sources such as glucose (Strauss et al., 1995).

In *T. reesei* and other fungi, the key regulator of CCR is the Cys2His2-type transcription factor CRE1. The functional binding site for this transcription factor (TF) consists of two closely spaced 5′-SYGGRG-3′ motifs. It has been suggested that CRE1-mediated repression requires both binding sites (Kubicek et al., 2009). Binding of CRE1 to DNA may be regulated by phosphorylation of a serine in a short, conserved region of CRE1 by casein kinase 2 protein (Cziferszky et al., 2002). However, we have demonstrated that there is no correlation between gene expression and the number of putative CRE1 binding sites in the promoter regions of cellulolytic and xylanolytic genes (Castro et al., 2014).

CRE1 is similar to other fungal proteins that mediate CCR, such as CreA in *Aspergillus nidulans* and Mig1 in *Saccharomyces cerevisiae* (Portnoy et al., 2011a). In the hypercellulolytic *T. reesei* strain

Rut-C30, which produces cellulases and hemicellulases in medium containing glucose, a truncated form of *cre1* has been identified (Peterson and Nevalainen, 2012). In the Rut-C30 strain, complementation of this mutation with wild-type *cre1* restored catabolite repression in the presence of glucose, providing further evidence for the repressive role of CRE1 (Ilmen et al., 1996). In the yeast *S. cerevisiae*, Mig1 represses the transcription of about 90 genes associated with the use of alternative carbon sources such as sucrose, galactose, and maltose under conditions when glucose is sufficient (Santangelo, 2006).

CCR presents a major challenge to the biotechnology industry for the production of cellulolytic enzymes because the presence of glucose in the culture medium suppresses the production of these enzymes. Thus, there is an ongoing search for ways to circumvent this mechanism and thereby improve the application of cellulases in several industrial sectors, mainly in the production of second generation bioethanol. Despite the current understanding of CRE1-mediated CCR in readily available carbon sources such as glucose, little is known about how this TF acts in the presence of more complex carbon sources, such as cellulose. In the present study, to evaluate CRE1-mediated CCR, we used an RNA sequencing (RNA-Seq) approach to perform a large-scale comparative analysis of the transcriptomes of *T. reesei* strains QM9414 and Δ*cre1* grown in the presence of cellulose and glucose. Our results showed that deletion of the *cre1* gene in *T. reesei* altered the expression of genes related to oxidoreductase activity, nutrient transport, carbohydrate metabolism, and transcription regulation, among others. Gene expression was regulated in a carbon-dependent manner, mostly indirectly.

## 2. Material and methods

### 2.1. Strains and growth conditions

*T. reesei* strains QM9414 (ATCC 26921) and Δ*cre1* (Portnoy et al., 2011a) were obtained from the Institute for Chemical Engineering (Vienna University of Technology, Research Area Gene Technology and Applied Biochemistry, Vienna, Austria). The strains were maintained on MEX (malt extract 3% (*w/v*) and agar–agar 2% (*w/v*)) medium at 4 °C. Both strains were grown on MEX at 28 °C from days 7–10 until the complete sporulation. For the RNA-Seq experiments, a spore suspension (NaCl 0.8% (*w/v*), Tween 80 0.05% (*v/v*)) of each strain was inoculated in a 1-L Erlenmeyer flask containing 200 mL of Mandels-Andreotti medium (Schmoll et al., 2009) containing 1% (*w/v*) of cellulose (Avicel) or 2% (*w/v*) of glucose, depending of the experiment. The flasks were incubated at 200 rpm and 28 °C for 24, 48, and 72 h when grown in cellulose medium and for 24 and 48 h when grown in glucose medium. For real-time quantitative PCR (RT-qPCR) experiments, a spore suspension of strain QM9414 or Δ*cre1* was inoculated in a 250-mL Erlenmeyer flask containing 25 mL of Mandels-Andreotti medium containing 2% (*w/v*) of glucose. After 48 h, the pre-grown mycelia were collected, washed three times with Mandels-Andreotti medium without a carbon source, and transferred to 25 mL of Mandels-Andreotti medium containing 1% (*w/v*) of cellulose. The mycelia were collected at 6, 12, and 24 h, or 1% (*w/v*) of glucose was added after 24 h of growth, and the mycelia were collected 6, 12, and 24 h later. All experiments were conducted in biological triplicates for each sample. After induction, the mycelia were collected by filtration, frozen, and stored at -80 °C.

### 2.2. RNA extraction

The mycelia of *T. reesei* strains QM9414 and Δ*cre1*, grown in different carbon sources as described above, were filtered through Miracloth, frozen in liquid nitrogen, and macerated. Total RNA was extracted using TRIzol® RNA reagent (Life Technologies) according to the manufacturer's instructions. RNA was quantified, and the integrity was checked using a 2100 Bioanalyzer (Agilent).

### 2.3. RNA-Seq

Total RNA of three biological replicates obtained from *T. reesei* strains QM9414 and Δ*cre1* in presence of cellulose (24, 48 and 72 h) and glucose (24 and 48 h) were used for sequencing. The total RNA from three time points were pooled, lyophilized and stored using RNAstable Tube kit (Biomatrica) to stabilize the RNA for sequencing. The barcoded libraries were prepared and sequenced by LGC Genomics GmbH (Berlin/Germany) using the Illumina Hiseq 2000 platform.

### 2.4. Data analysis

Sequences were mapped based on the reference genome of *Trichoderma reesei* 2.0, obtained from the Joint Genome Institute (JGI) Genome Portal (http://genome.jgi-psf.org/Trire2/Trire2.home.html), using Bowtie version 0.12.8 (Li and Durbin, 2009), with only unique alignments allowed. After alignment, SAMtools version 0.1.18 (Li et al., 2009) was used to process the alignments files, which were visualized using the Integrative Genomics Viewer (Thorvaldsdottir et al., 2013). The Bioconductor DESeq package version 1.10.1 (Anders and Huber, 2010) was used for the differential expression analysis, and a twofold change cut-off ($\log_2$ fold change) of $\geqslant 1$ or $\leqslant -1$ and an adjusted *p*-value of $\leqslant 0.05$ were established as thresholds. Samples were normalized using median log deviation implemented in the DESeq package. Functional categorization was performed with Gene Ontology (GO) terms using BayGO software (Vencio et al., 2006), adopting a *p*-value of $\leqslant 0.05$ as the criterion for significantly enriched categories. The network of genes upregulated and downregulated by CRE1 in different carbon sources was generated using Cytoscape version 3.0.1 (Shannon et al., 2003). Raw sequence data and count data for all samples are available at GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE57374 and GSE53629. Protein sequences from *T. reesei* and other species used in the phylogenetic analysis were obtained from the JGI Genome Portal and other online databases, respectively. Multiple sequence alignment was performed using ClustalW, and neighbor-joining trees were created with the Mega 4 program (Tamura et al., 2007) using 1000 bootstraps.

### 2.5. RT-qPCR

To validate the differentially expressed genes, we used the pooled RNA samples from strains QM9414 and Δ*cre1* grown in cellulose. Twenty genes were used for validation, including hydrolytic enzymes and other proteins chosen randomly. In this analysis, we used the sample from QM9414 grown in cellulose as the reference. In experiments evaluating the expression of the genes ID 3405, ID 48444, ID 52315, and ID 111750 in QM9414 and Δ*cre1* strains grown on cellulose and then transferred to glucose (see Section 2.1), we used the absolute gene expression values with the actin gene as an endogenous control. The primers used in these experiments are described in Table S1.

For these experiments, 1 μg of RNA was treated with DNase I (Fermentas) to remove genomic DNA. cDNA was then synthesized using a Maxima First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions. The cDNA, diluted 1:50, was analyzed with real-time PCR using the CFX96™ Real-Time PCR Detection System (Bio-Rad) and the SsoFast™ EvaGreen® Supermix (Bio-Rad), in accordance with the manufacturer's instructions. The actin gene was used as the endogenous control