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Trichoderma reesei CRE1-mediated Carbon Catabolite Repression in Response to Sophorose Through RNA Sequencing Analysis

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Abstract: Carbon catabolite repression (CCR) mediated by CRE1 in *Trichoderma reesei* emerged as a mechanism by which the fungus could adapt to new environments. In the presence of readily available carbon sources such as glucose, the fungus activates this mechanism and inhibits the production of cellulolytic complex enzymes to avoid unnecessary energy expenditure. CCR has been well described for the growth of *T. reesei* in cellulose and glucose, however, little is known about this process when the carbon source is sophorose, one of the most potent inducers of cellulase production. Thus, we performed high-throughput RNA sequencing to better understand CCR during cellulase formation in the presence of sophorose, by comparing the mutant Δ*cre1* with its parental strain, QM9414. Of the 9129 genes present in the genome of *T. reesei*, 184 were upregulated and 344 downregulated in the mutant strain Δ*cre1* compared to QM9414. Genes belonging to the CAZy database, and those encoding transcription factors and transporters are among the gene classes that were repressed by CRE1 in the presence of sophorose; most were possible indirectly regulated by CRE1. We also observed that CRE1 activity is carbon-dependent. A recent study from our group showed that in cellulose, CRE1 repress different groups of genes when compared to sophorose. CCR differences between these carbon sources may be due to the release of cellodextrins in the cellulose polymer, resulting in different targets of CRE1 in both carbon sources. These results contribute to a better understanding of CRE1-mediated CCR in *T. reesei* when glucose comes from a potent inducer of cellulase production such as sophorose, which could prove useful in improving cellulase production by the biotechnology sector.

Keywords: *Trichoderma reesei*, Carbon catabolite repression, CRE1, Sophorose, RNA-seq.

1. INTRODUCTION

The genus *Trichoderma* includes soilborne, green spored ascomycete fungi found all over the world [1]. One of the best studied fungi species in this genus is *Trichoderma reesei*, which is used in the biotechnology industry as a source of cellulases and xylanases for biomass degradation [2]. Therefore, this fungus might be employed as an excellent model for studies of other fungal cellulolytic systems [3].

High levels of cellulase and hemicellulase gene expression can be achieved during *T. reesei* growth in cellulose, xylan, lactose or a mixture of plant polymers [4, 5]. Sophorose, a natural compound, is the strongest known cellulase inducer in this organisms and is formed by the transglycosylation of cellobiose during cellulose hydrolysis [6]. Furthermore, induction by sophorose results in higher cellulase expression levels compared to cellulose in the same period of time [7]. Nevertheless, other compounds such as L-arabitol and L-sorbose have also been reported to promote cellulase gene expression [8]. By contrast, easily metabolizable carbon sources such as glucose repress the expression of cellulolytic genes [9].

The regulation of cellulase and hemicellulase gene expression has been extensively investigated in *T. reesei* [10, 11]. This process is controlled by several transcription factors such as the positive regulators ACE2, ACE3, XYR1, and HAP2/3/5, and the negative regulators ACE1 and the carbon catabolic repressor CRE1. [12, 13]. Recently, it was demonstrated that the regulation of cellulase and xylanase expression by CRE1 and XYR1 occurs in a carbon source-dependent manner [14]. Carbon catabolic repression (CCR) in *T. reesei* is controlled by the transcription factor CRE1 [15]. CRE1 may be regulated by casein kinase 2 through the phosphorylation of a serine residue situated in a conserved region of this transcription factor, a process which is required for CRE1 to bind to a specific DNA fragment [16]. The gene expression regulation promoted by CRE1 in *T. reesei* may occur at different levels. CRE1 showed to be present in the nucleus in the presence of glucose and may

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bind in inverted and direct repeats in upstream regulatory gene regions [17-20]. It also regulates the expression of XYR1 (the main transactivator of plant cell wall degrading enzyme expression in the presence of D-xylose) [21-23] and finally, act in the nucleosome positioning on promoters of different genes, such as *cbh1* and *cbh2* [24, 25].

Portnoy *et al.* demonstrated that CRE1-mediated CCR can lead to the repression of genes related to nitrogenous substance uptake, components of chromatin remodeling and the transcriptional mediator complex, as well as genes associated with developmental processes in glucose [26]. Recently, our group investigated CCR during the synthesis of cellulases, comparing the *T. reesei* mutant strain Δ*cre1* with its parental strain QM9414 during glucose and cellulose induction [23]. In this study, we have shown that genes encoding cellulolytic enzymes and transcription factors, as well as genes related to the transport of nutrients and oxidative metabolism, were targets of CCR, mediated by CRE1 in a carbon source-dependent manner. Despite this knowledge about CCR in the presence of glucose and cellulose, the role of sophorose in the regulation of catabolic repression remains unclear. Here, we describe the use of RNA sequencing (RNA-seq) to perform a large-scale comparative analysis of the transcriptomes of *T. reesei* strains QM9414 and Δ*cre1* grown in the presence of sophorose. Our results shows that deletion of the transcription factor *cre1* in *T. reesei* alters the expression of genes belonging to CAZy, transcription factors and transporters in the presence of sophorose. We also demonstrated differences in CRE1 target genes when glucose arises from sophorose compared to glucose arising from cellulose derived-cellodextrins, and that most CRE1 targets seemed to be indirectly regulated by CRE1.

2. MATERIAL AND METHODS

2.1. Strains and Growth Conditions

T. reesei strains QM9414 (ATCC 26921) and Δ*cre1* [26] 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 7–10 days until the completion of sporulation. For the RNA-Seq experiments, a spore suspension containing approximately 10^7 spores/mL of each strain was previously inoculated in 200 mL of Mandels-Andreotti medium [27] containing 1% glycerol for 24 hours. Then, one quarter of the produced mycelium was washed with Mandels-Andreotti medium without peptone and carbon source and transferred into 20 mL of Mandels-Andreotti medium without peptone containing 1 mM of sophorose. The cultures were incubated on an orbital shaker (200 rpm) at 28°C for 2, 4 and 6 hours. The experiments were conducted in triplicate for each sample. After induction, the mycelia were collected by filtration, frozen and stored at -80°C until RNA extraction.

2.2. RNA Extraction

The mycelia of *T. reesei* strains QM9414 and Δ*cre1* grown in sophorose (2, 4 and 6 hours) were filtered through Miracloth, frozen in liquid nitrogen, and macerated. Total RNA was extracted using TRIzol® RNA reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was quantified and the integrity checked using a 2100 Bioanalyzer (Agilent Technologies, Waldbroon, Germany).

2.3. RNA-Seq

For sequencing, the total RNA obtained from the three biological replicates of QM9414 and Δ*cre1* in sophorose for 2, 4 and 6 hours was pooled, lyophilized and stored using an RNAstable Tube kit (Biomatrica, San Diego, CA, USA) 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

The Illumina Hiseq 2000 system was used to sequence approximately 76 million 100 bp paired-end reads. Sequences were mapped based on the reference genome of *T. 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 [28], with only unique alignments allowed. After alignment, SAMtools version 0.1.18 [29] was used to process the alignment files, which were visualized using the Integrative Genomics Viewer [30]. The Bioconductor DESeq package version 1.10.1 [31] was used for differential expression analysis, and a two-fold change cut-off (log2 fold change) of ≥ 1 or ≤ -1 and an adjusted p-value of ≤ 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 [32], adopting a p-value of ≤ 0.05 as the criterion for significantly enriched categories. Raw sequence data and count data for all samples are available at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE66915 and GSE53629. Protein sequences from *T. reesei* and the other species used in phylogenetic analysis were obtained from the JGI Genome Portal and other online databases, respectively. Multiple sequence alignment was performed using ClustalW, and Maximum Parsimony trees were created using the Mega 4 program [33] with 1000 bootstraps.

2.5. Quantitative Real-time PCR For Validation of RNA-seq Data

To validate the differentially expressed genes, we used the pooled RNA samples from strains QM9414 and Δ*cre1* grown in sophorose. Nineteen genes were used for validation, including those encoding hydrolytic enzymes and other randomly chosen proteins. In this analysis, we used the sample from QM9414 grown in sophorose as the reference. The primers used are described in (Table **S1**). For quantitative real-time PCR (qRT-PCR), 1 μg of RNA was treated with DNase I (Fermentas, Waltham, Massachusetts, USA) to remove genomic DNA. cDNA was subsequently synthesized using a Maxima First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The cDNA was diluted 1:50 in water and analyzed with qRT-PCR using the CFX96™ Real-

Time PCR Detection System (Bio-Rad, San Francisco, CA, USA) and the SsoFast™ EvaGreen® Supermix (Bio-Rad), in accordance with the manufacturer's instructions. The actin gene was used as the endogenous control to normalize the total amount of cDNA present in each reaction. The amplification program used in this study consisted of: 95°C for 10 minutes; 39 cycles of 95°C for 10 seconds and 60°C for 30 seconds; and a dissociation curve of 60°C to 95°C at increments of 0.5°C for 10 seconds. Gene expression levels were calculated as described by Livak and Schmittgen [34].

2.6. *In Silico* Analysis of Putative CRE1 Binding Sites

For *in silico* prediction of CRE1 binding sites, 1.5 kb sequences upstream of the ATG start codon in the analyzed genes were retrieved from the genomic sequence of *T. reesei*, available in the JGI *T. reesei* database (http://genome.jgi-psf.org/Trire2/Trire2.home.html). Double binding motifs for CRE1, defined as inverted or direct repeats of the core consensus sequence, were identified in the promoter regions using 9.0 as the threshold [19]. CRE1 binding motif is represented in the (Fig. **S1**). Genes showing binding sites for CRE1 were considered possible directly regulated by this transcription factor and are underlined in the Tables **1**, **2** and **3** and supplementary Tables **S3** and **S4**.

3. RESULTS

3.1. Deep RNA Sequencing of *T. reesei* and Transcriptome Analysis

To identify the role of the transcription factor CRE1 in the global transcriptome of *T. reesei* during growth in the presence of sophorose, we performed high-throughput RNA-sequencing using the Illumina/HiSeq2000. *T. reesei* strains QM9414 and Δ*cre1* were first grown in a medium containing glycerol and then transferred to a medium containing sophorose as the sole carbon source, as described in Material and methods. Three biological replicates of each condition were sequenced, yielding approximately 76 million 100 bp paired-end reads, corresponding to 15 GBs of nucleotides. Reads were uniquely mapped to the *T. reesei* QM6a reference genome available from JGI (*Trichoderma reesei* 2.0) using the Bowtie aligner. On average, 70% of the total number of reads was aligned to the *T. reesei* reference genome (Table **S2**). The three biological replicates of each condition showed a high Pearson correlation ($R \geq 0.84$) and the Principal Component Analysis demonstrated the reliability of RNA-seq (Fig. **S2** and **S3**, respectively).

The Bioconductor DESeq package was used to perform differential expression analysis in the mutant Δ*cre1* compared to QM9414 and found 2368 genes modulated by CRE1 in the presence of sophorose, using a p-value ≤ 0.05 as the threshold (Fig. **1A**). Of these, 184 were upregulated and 344 were downregulated, using a two-fold change cut-off (log2 fold change) of ≥ 1 or ≤ -1, respectively, and an adjusted p-value of ≤ 0.05 as the threshold (Fig. **1B**).

3.2. Genes Regulated By CRE1 in the Presence of Sophorose

Of 9129 genes in the *T. reesei* genome, 528 genes were up- or downregulated in the mutant strain when growing in sophorose (Table **S3**). To simplify the analysis, we selected the top 15 differentially expressed genes (Table **1**). Among these, the top 15 upregulated genes included the glycoside hydrolase (GH) GH71 (ID 120873) and five transport proteins (ID 76682, 123718, 102851, 123079 and 76971). Genes that encode a unique protein (ID 119857), Epl1/Sm1 (ID 123955), OOC1 (ID 50323) and two unknown proteins (ID 33827 and ID 55887) were also upregulated in the Δ*cre1* mutant in the presence of sophorose, as well as genes related to a transcription factor (ID 3449), one protein involved with fatty acid biosynthesis (ID 82208), one in the response to antibiotic (ID 58717) and one dehydrogenase (ID 69692). Among these genes, *Epl1/Sm1* (ID 123955) was the only that seems to be possible directly regulated by CRE1.

To categorize the genes upregulated in the Δ*cre1* mutant, we used classification according to GO terms (Fig. **2**). Categories such as “integral to membrane” (12%), “transport” (11%), “membrane” (9%), “transporter activity” (7%) and “carbohydrate metabolic process” (7%) were significantly enriched among the upregulated genes. Furthermore, CCR triggered by CRE1 represses some genes encoding hydrolytic enzymes required for the hydrolysis of lignocellulosic material (Table **S4**). This is demonstrated in our results since during sophorose cultivation, genes related to the metabolism and transport of carbohydrate and hydrolytic activity were upregulated in the absence of *cre1* (Fig. **2**).

3.3. Expression of Carbohydrate-active Enzyme (CAZy) Genes

The enzymes collectively called CAZy synthesize and degrade carbohydrates for a range of biological functions. In 2012, Häkkinen *et al*. [35] performed a re-annotation of all the genes encoding enzymes belonging to this CAZy group in *T. reesei*. In doing so, they identified 201 genes encoding glycosyl hydrolases, 22 for carbohydrate esterases and 5 for polysaccharide lyases. Based on these results, we selected all the genes belonging to CAZy which are under CRE1-mediated CCR in the presence of sophorose, considering a p-value ≤ 0.05 as the threshold (Fig. **3** and Table **S4**).

We found 22 genes upregulated in the mutant Δ*cre1* compared to QM9414, among which 19 are classified as glycosyl hydrolases and 3 as carbohydrate esterases. The candidate α-1,3-glucanase gene (ID 120873) reached the highest fold change (Log_2 fold change = 4.71), followed by *GH54* (ABF1) (Log_2 fold change = 2.61), *GH2* (β-mannosidase) (Log_2 fold change = 2.49) and *CE5* (cutinase) (Log_2 fold change = 2.34). It is interesting to note that only two CAZy genes seemed to be possible directly regulated by CRE1 (ID 79921 and 122081), reinforcing the idea that CRE1 gene expression regulation likely involves a net of transcription factors and not only CRE1.

3.4. Expression of Transcription Factors

Transcription factors (TFs) are proteins involved in the regulation of several metabolic processes occurring in the cellular environment. For instance, the production of cellulases in the presence of sophorose requires the action of many TFs, with some already characterized but others are still undescribed. Thus, we investigated how CCR mediated by CRE1 in the presence of sophorose influences in the 493

Table 1. Top genes under CRE1-mediated CCR during *T. reesei* growth in presence of sophorose. Gene expression values are represented as Log_2 fold change. Genes directly regulated by CRE1 are underlined in the table.

Protein ID	Description	FC	p-value
76682	PDR-type ABC transporters	6.0	2.0E-05
120873	GH71 α-1,3-glucanase	4.7	6.4E-245
119857	unique protein	4.0	2.8E-78
69692	Quinoprotein amine dehydrogenase beta chain-like protein	3.6	3.0E-136
123955	Epl1/Sm1	3.4	1.8E-25
102851	SSCRP	3.3	1.3E-74
123718	amino acid transporter, neutral 11 TM	3.3	2.7E-89
50323	OOC1	3.1	7.9E-07
3449	Zn2Cys6 transcriptional regulator	3.1	1.6E-36
76971	SSCRP	2.9	2.9E-44
58717	ß-lactamase class C	2.9	2.9E-19
33827	unknown protein	2.9	1.3E-13
55887	unknown protein, secreted	2.9	7.6E-37
123079	short chain dehydrogenase/reductase	2.8	5.6E-116
82208	PKS	2.8	5.9E-23

Table 2. Transcription factor genes upregulated in the mutant Δ*cre1* compared to QM9414 in presence of sophorose. Gene expression values are represented as Log_2 fold change. Genes directly regulated by CRE1 are underlined in the table.

Protein ID	Description	FC	p-value
3449	Zn2Cys6 transcriptional regulator	3.11	1.6E-36
4124	myb transcriptional regulator	2.40	2.7E-28
121415	Zn2Cys6 transcriptional regulator	1.98	5.9E-25
108357	C2H2 transcriptional regulator	1.50	9.3E-07
123713	transcriptional regulatorMedA, involved in fruiting body development	1.44	7.1E-27
58011	C2H2 conidiation transcription factor FlbC	1.39	9.6E-26
26871	Zn2Cys6 transcriptional regulator	1.28	3.6E-15
109328	Zn2Cys6 transcriptional regulator	1.02	5.0E-13

Table 3. Transporter genes upregulated in the mutant Δ*cre1* compared to QM9414 in presence of sophorose. Gene expression values are represented as Log_2 fold change. Genes directly regulated by CRE1 are underlined in the table.

Protein ID	Description	FC	p-value
76682	PDR-type ABC transporter	6.03	2.0E-05
123718	amino acid transporter, neutral 11 TM	3.33	2.7E-89
65191	MFS permease (maltose permease)	2.65	4.9E-77
104072	xylose transporter	2.64	1.1E-73

(Table 3) contd….

Protein ID	Description	FC	p-value
69834	MFS permease	2.57	4.1E-72
82105	PDR-type ABC transporters	2.44	2.9E-02
121482	MFS permease	2.42	4.4E-82
62488	MFS permease	2.39	1.6E-44
80058	MFS permease	2.16	1.4E-19
62172	amino acid permease (PotE?)	2.12	5.2E-44
65915	MFS permease	1.85	1.3E-36
123293	MDR-type ABC transporters	1.67	3.5E-03
71029	Ctr copper transporter, putative	1.42	4.5E-21
104320	MFS permease	1.41	4.7E-11
80639	iron transporter	1.40	7.6E-11
73924	MDR-type ABC transporters	1.37	5.9E-19
55077	MFS permease	1.34	2.3E-20
58899	MDR-type ABC transporters	1.24	3.0E-02
41761	iron transporter	1.19	1.5E-14
68869	MFS permease	1.16	4.1E-14
76897	MFS permease	1.08	1.8E-14
59515	MFS permease	1.02	7.2E-13

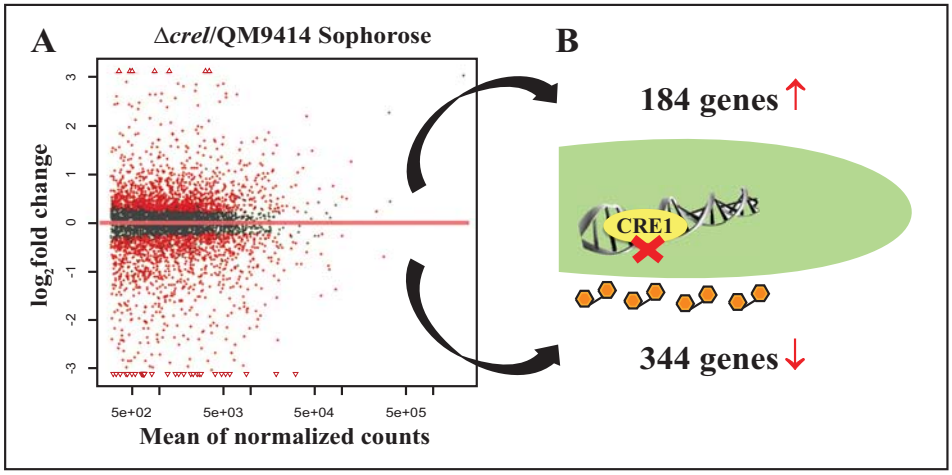

Fig. (1). Differentially expressed genes in the mutant Δ*cre1* relative to the parental strain QM9414. The expression profile was calculated for conditions inlcuding sophorose. Genes that showed differential expression identified by DESeq package are shown in red. Among the 9129 *T. reesei* genes, 2368 were differentially expressed, using a p-value value ≤ 0.05 as threshold (**A**). Of these, 184 genes were upregulated and 344 genes were downregulated in the mutant Δ*cre1* compared to QM9414 (**B**).

TFs (information based on the number of *T. reesei* transcription factors annotated in the genome portal http://genome.jgi-psf.org/Trire2/Trire2.home.html) found in the genome of *T. reesei*.

Eight TF genes were subject of CCR by CRE1 in the presence of sophorose (Table **2**), five of which are proteins with yet undefined function. The most expressed gene coding a TF in the strain Δ*cre1* as compared to QM9414 is a Zn2Cys6 type TF (ID 3449). It was almost nine times (Log_2 fold change = 3.11) more heavily expressed in the mutant Δ*cre1* compared to the parental strain. The second most repressed TF gene in the presence of sophorose by CRE1

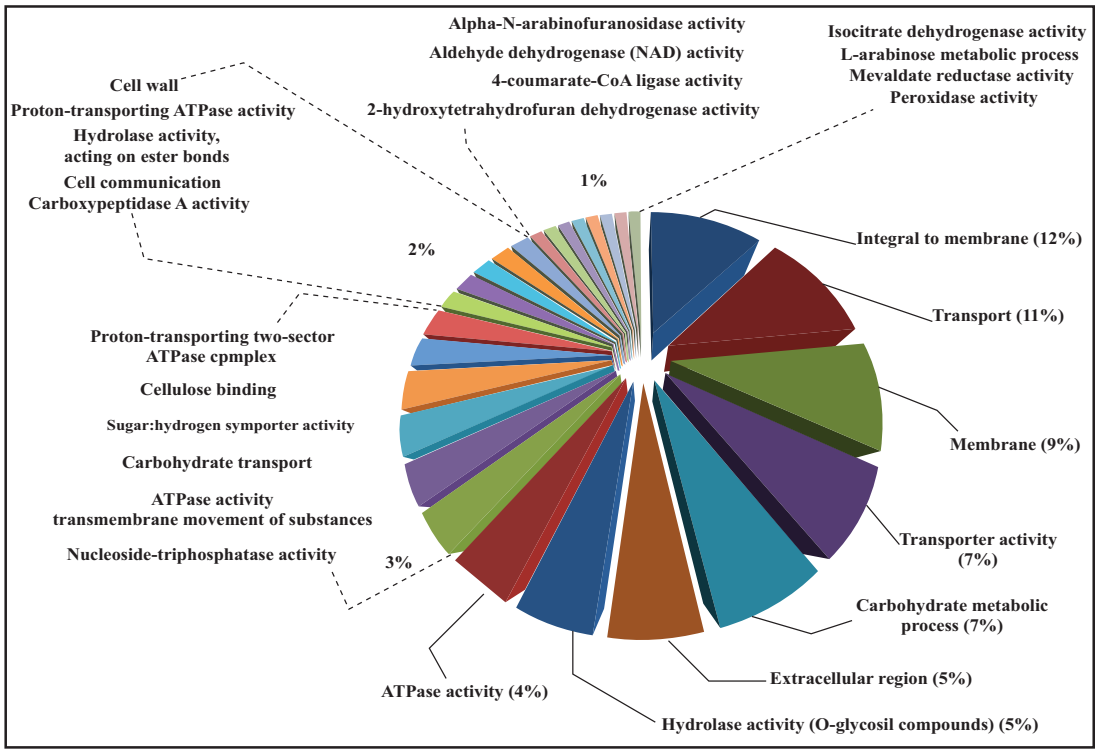

Fig. (2). Enrichment analysis using Gene Ontology terms of the major genes upregulated in Δ*cre1* compared to QM9414 during growth in sophorose. Functional categorization was performed using BayGO software [32] and the categories were considered significantly enriched when $p \leq 0.05$.

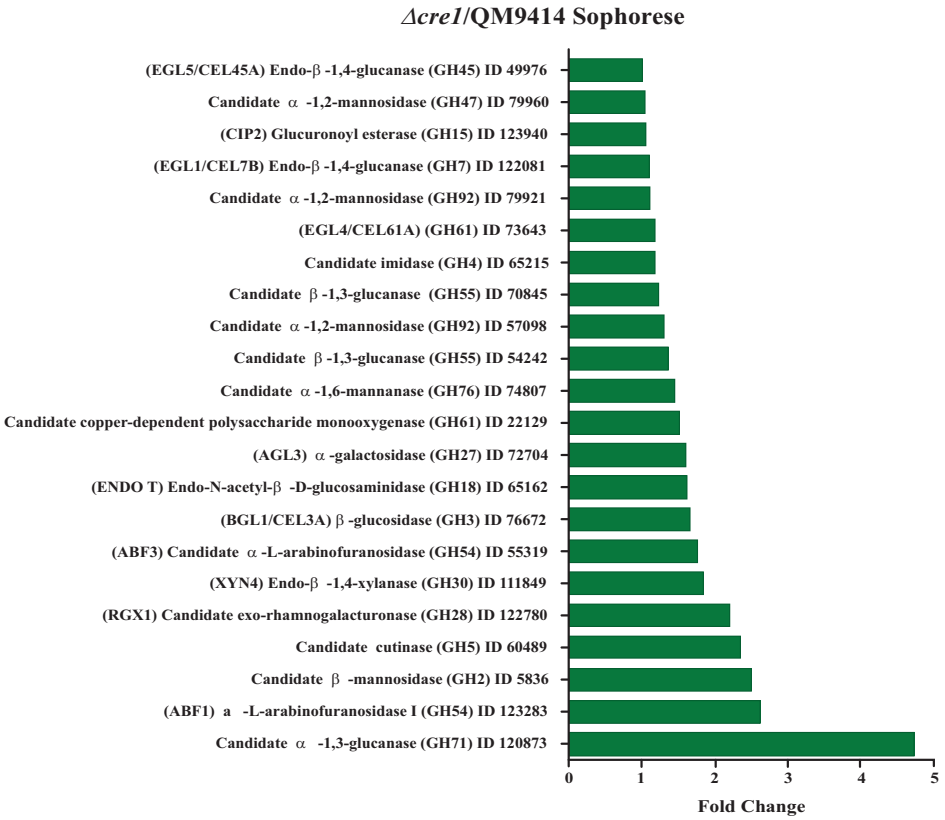

Fig. (3). CAZy genes upregulated in the mutant Δ*cre1* compared to QM9414 in the presence of sophorose. Gene expression values are represented as Log_2 fold change.

belongs to the Myb family (ID 4124; Log_2 fold change = 2.40). Another Zn2Cys6 TF gene (ID 121415) with unknown function ranks third among the regulatory proteins under CCR in sophorose, which was four times more heavily expressed in the Δ*cre1* strain compared to QM9414 (Log_2 fold change = 1.97). Other TF genes that were repressed by CRE1 in the presence of sophorose include the transcriptional regulator MedA (ID 123713) and the conidiation TF FlbC (ID 58011). The latter is the only TF with predicted binding sites for CRE1. Together, these data suggest that CRE1-mediated CCR in the presence of sophorose can occur indirectly through the recruitment of other TFs, which assist in the process of cellulase production by *T. reesei*.

3.5. Expression of Transporters

The *T. reesei* genome is composed of 459 genes encoding proteins with a transport function (information based on the number of *T. reesei* transporters annotated in the genome portalhttp://genome.jgi-psf.org/Trire2/Trire2.home.html), which comprises approximately 5% of the total genome of the fungus. Due to this large number of transporters, we investigated the relationship between the gene expression and CRE1-mediated CCR during *T. reesei* growth in the presence of sophorose.

We found 22 transporters to be upregulated in the Δ*cre1* strain compared to QM9414 (Table **3**). Half of these genes encode proteins that belong to the Major Facilitator Superfamily (MFS) permeases. However, the gene most repressed by CRE1 in the presence of sophorose encodes a PDR-type ABC transporter (ID 76682), which was 65 times (Log_2 fold change = 6.03) more expressed in the mutant Δ*cre1* when compared to the parental strain, QM9414. Four other ABC transporter genes (ID 82105, 123293, 73924 and 58899) were repressed by CRE1 in the presence sophorose. The second most repressed gene by CRE1 in the described condition encodes an amino acid transporter (ID 123718), which was 10 times (Log_2 fold change = 3.33) more expressed in the mutant than in the parental strain. Ion transporters, such as iron and copper, are also among the major transport proteins repressed by CRE1 in the presence of sophorose. Among the transporters, only the MFS permease (ID 55077) was predicted as possible directly regulated by CRE1.

In order to better understand the function of each target transporter of CRE1-mediated CCR in the presence of sophorose, we performed a phylogenetic analysis using the amino acid sequences of the transporters upregulated in Δ*cre1* compared to QM9414. The transport-related proteins of different species were also included in the analysis (Table **S5**).

We observed the formation of five clusters in the phylogenetic tree: two composed of ABC transporters and three related to MFS permeases, maltose permeases, and sugar transporters (Fig. **4**). In the largest cluster of sugar transporters, we identified two MFS permeases and a xylose transporter from *T. reesei* that are similar to 11 sugar transporters from other species, suggesting that these *T. reesei* transporters are involved in the uptake of sugars into the cell. A second cluster composed of maltose permeases contained two MFS permeases form *T. reesei*, one of which has previously been described as a MFS maltose permease. Along with other maltose and sugar transporters from *Aspergillus oryzae*, *Talaromyces marneffei*, *Metarhizium anisopliae,* and *Talaromyces stipitatus*, they formed a group of proteins that are likely involved in the transport of disaccharides, such as cellobiose or sophorose, which are produced as a result of the degradation of the cellulose polymer by the cellulolytic complex. Six MFS permeases from *T. reesei* formed a third cluster and finally, two groups of ABC transporters were found. The first was composed of three *T. reesei* ABC transporters and an ABC transporter from *A. niger*. The other group comprises two *T. reesei* ABC transporters and three from *Colletotrichum gloeosporioides*, *Togninia minima*, and *M. anisopliae*.

3.6. Validation of RNA-seq Data

We performed qRT- PCR to validate RNA-seq data in order to ensure the viability of our results and proceed with further analyzes. The expression of 19 genes was analyzed using Δ*cre1* and QM9414 strains grown in sophorose. Among the tested genes, those encoding hydrolytic enzymes were analyzed, as well as other randomly chosen proteins. The results of the qRT-PCR experiments are shown in Table **S6**. As can be observed, only one gene showed no correlation between the qRT-PCR and the RNA-seq data. A high correlation was found between the two techniques (r^2 = 0.96), reflecting the quality of the chosen method and the manner in which both experiments were conducted (Fig. **S4**).

4. DISCUSSION

CRE1-mediated CCR in the fungus *T. reesei* emerged as a system of adaptation to the nutrients available in the environment. Several reports have shown that different genes are induced or repressed by CRE1 in the presence of carbon sources of variable complexity, such as cellulose, sophorose and glucose [14, 23, 26]. Furthermore, we observed that CRE1 regulates the expression of its target genes in a carbon source-dependent manner [21].

Initially, it was believed that CRE1-mediated CCR occurred by the binding of the TF to the promoter region of several genes that comprise the cellulolytic complex, thereby blocking the production of cellulase when a readily metabolizable carbon source was present in the medium [15]. While this remains correct, recent studies have demonstrated that CRE1 may also control genes related to several other functions in *T. reesei* and only a limited number of the CAZy genes are direct targets of this TF during CCR [23, 26]. We found a similar result during growth of the *T. reesei* Δ*cre1* in sophorose, in which most of the CAZy genes were not repressed by CRE1 and some genes were even stimulated by this TF (data no shown).

The gene coding for the β-glucosidase BGL1/Cel3a (ID 76672) is known to be strongly induced in the presence of sophorose [36]. In the present study, we found that this CAZy gene was repressed by CRE1, in accordance with its function to hydrolyze sophorose into two glucose molecules [37], thus an attractive target for CRE1 when glucose begins to appear in the culture medium. Castro *et al*. [38] also described a higher transcriptional induction of cellobiohydrolase genes members from the families GH6 and GH7 in the presence of cellulose and sophorose. Among these, 20 GHs

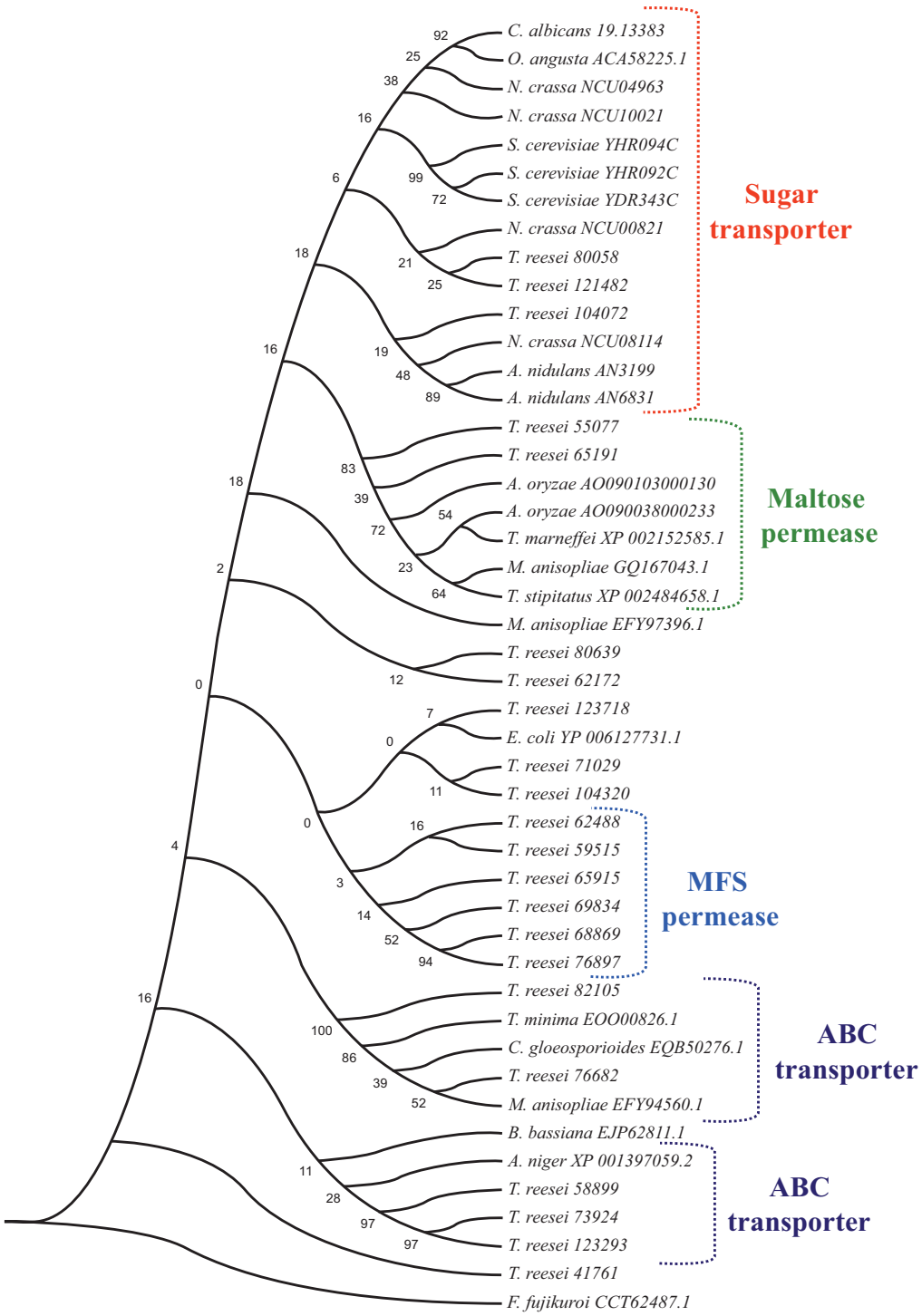

Fig. (4). Phylogenetic analysis of transporters upregulated in the mutant Δ*cre1* during growth in sophorose. The analysis also includes other proteins involved with transport in different species. Highlights: five clusters formed by MFS permeases, maltose permeases, ABC transporters and sugar transporters. The tree was created using the Mega 4 program using the Maximum Parsimony method with 1000 bootstraps.

and 1 CE were upregulated in the QM9414 strain in response to sophorose. Here, we describe 19 genes encoding GHs (mainly GH 54, GH 55, GH 61, and GH 92) and three encoding CEs (ID 60489, ID 65215 and ID 123940) that are subject to CRE1-mediated CCR. Interestingly, the genes involved in arabinoxylan degradation (the arabinofuranosidases *abf1* and *abf3*) and xylan degradation (*xyn4*), previously thought to be expressed only in cellulose, were upregulated in the Δ*cre1* strain grown in the presence sophorose (Table **S4**). These results suggest that CRE1 is responsible for the CCR of these genes in the presence of sophorose as well as less complex carbon sources. Recently, our group showed that CRE1-mediated CCR predominantly inhibits genes related to oxidative metabolism when in the presence of cellulose, affecting the early stages of cellulose polymer degradation [23]. The gene coding for the copper-dependent

enzyme polysaccharide monooxygenase GH61 (egl4/cel61a/AA9) ID 73643, one of the enzymes responsible for this initial oxidative metabolism of cellulose [39] was not, however, a target of CRE1-mediated CCR in this condition. However, we observed that this gene was expressed more than twice as heavily in the mutant strain Δ*cre1* as compared to QM9414 in sophorose, indicating that CRE1-mediated CCR also occurs in the early stages of degradation of the lignocellulosic polymer in this carbon source, but through different targets. This distinct regulation of CRE1-mediated CCR between cellulose and sophorose CRE1 can be explained by the fact that cellulose degradation releases, besides sophorose, cellodextrin molecules (glucose polymers such as cellobiose, cellotriose and cellotetraose) that are important in cellulase induction, as is the case in *N. crassa* and *Penicillium oxalicum* [40-42]. Glucose produced from cellodextrins likely signals to CRE1 through a different mechanism than occurs in sophorose, and therefore the CRE1-target genes are different in both conditions. Other genes related to this initial lignocellulosic polymer degradation stage that were repressed by CRE1 in sophorose are the copper and iron transporters. Copper is a cofactor required for the maximum activity of GH61 [39] and ferrous iron is required for the Fenton reaction that produces hydroxyl radicals from the reduction of extracellular metal ions randomly oxidizing cellulose [43]. Studies by Bischof *et al*. [44] showed that genes related to iron homeostasis are overexpressed in the carbon source wheat straw compared to glucose in *T. reesei*, making them interesting CRE1-targets when glucose derived from inducing sources, such as sophorose, begin to appear in the medium. These data suggest a similar mechanism of CRE-mediated CCR between cellulose and sophorose, in line with the fact that little difference is found between the gene expression and secretome of *T. reesei* QM9414 during growth in both conditions [38]. In addition to iron transporters, the ABC transporters represent another class of genes that were targeted for CCR in sophorose. The main CRE1 repressed gene in this condition, ID 76682, encodes a protein that contains an ABC transporter extracellular domain (Pfam 00005.22), which is found in proteins belonging to the ATP Binding Cassette (ABC) superfamily involved with the intracellular transport of metabolites across bacterial membranes [45, 46]. In fungi, these transporters have been shown to be involved with antifungal resistance and mycoparasitic interaction [47-50]. For example, Ruocco *et al*. [51] showed the role of an ABC transporter membrane pump in the interaction of *T. atroviride* with different plant pathogenic fungi. Interestingly, in the extreme thermoacidophilic archaeon *Sulfolobus solfataricus,* sugar transport is mediated by two families of protein binding dependent ABC transporters. Binding activity could be detected for arabinose, fructose, xylose, glucose, galactose, cellobiose, maltose, and trehalose [52]. Sophorose is formed by transglycosylation of cellobiose during cellulose hydrolysis [6], indicating a structural similarity between these sugars. Therefore, the upregulation of this transporter gene in Δ*cre1* grown in the presence of sophorose might indicate an additional mechanism of available sugar transport into the cell.

Genes coding for MFS permeases were also highly repressed by CRE1 in sophorose. Among them is the xylose transporter gene (ID 104072), strongly transcribed in wheat straw and repressed in glucose grown *T. reesei* [53]. Another study conducted by de Souza *et al*. [54] showed that the expression of transporter genes possibly involved in the transport of sugars was highly modulated in *A. niger* during growth in sugarcane bagasse. Additionally, a recent work has demonstrated that, in *T. reesei*, the transport of molecules by MFS permeases and ABC transporters is more strongly induced by wheat straw and lactose than a glucose carbon source [44, 55]. In *N. crassa*, transporter genes were also repressed by CRE1 in the presence of cellulose [56]. Together, these data suggest that sugar transporters are potent targets of the process of CRE1-mediated CCR, as observed in our results.

Our group has shown that the blocking of cellulase synthesis mediated by CRE1 does not necessarily occur by the direct binding of this TF to its target genes [23]. In most cases, CRE1 must alter the expression of other regulatory proteins to achieve such inhibition. Previous studies demonstrated that genes encoding transcription factors are highly repressed in the presence of glucose compared to lactose and wheat straw [44], indicating that these genes may be targets of CRE1, as observed in our results in the presence of sophorose. Among the TFs inhibited by CRE1 grown in sophorose is the *myb* gene, previously described as a regulator of nitrogen metabolism in *Aspergillus nidulans* [57] and *Fusarium graminearum* [58]. Regarding nitrogen metabolism, a gene encoding an amino acid transporter (ID 123718) was also repressed by CRE1 in sophorose. During growth in lactose however, it was demonstrated that genes involved in amino acid metabolism were more highly expressed compared to growth in glucose [55]. Therefore, we suggest that, in the presence of readily available carbon sources, *T. reesei* tends to block the use of nitrogen compounds as an alternative energy source. A gene encoding the transcriptional regulator MedA, involved in sporulation, was also repressed by CRE1 in sophorose. In another study with *T. reesei*, this gene was the target of CRE1 during growth in glucose at high growth rates [26]. The gene encoding the TF FlbC, which is involved in the process of conidiation in *A. nidulans*, was also inhibited by CRE1 in the presence of sophorose [59]. Thus, we suggest that asexual development in the fungus *T. reesei* can be affected by CRE1, a notion reinforced by studies showing that deletion of *cre1* results in reduced radial growth rate, smaller colonies and aerial hyphae and fewer spores in *T. reesei* and *N. crassa* [26, 56, 60]. Among the regulators with undefined function, the TF with gene ID 108357, upregulated in mutant Δ*cre1* grown in sophorose, was highly transcribed in *T. reesei* grown in the presence of wheat straw but repressed in *T. reesei* grown in glucose [53], corroborating our finding that it may be a target of CRE1-mediated CCR.

Moreover, we found an enrichment of genes encoding accessory proteins such as small secreted cysteine-rich protein (SSCRP ID 76971 and ID 102851), OCC1 (ID 50323) and Epl1 (ID 123955) in the mutant Δ*cre1* compared to QM9414 during growth in sophorose. Curiously, we observed overrepresented down-regulation of genes related to electron transport (ID 21876, ID 77288 and ID 73631) in the mutant Δ*cre1* (data not shown). These results suggest that, in the presence of sophorose, a decrease in oxidative metabolism occurs in the Δ*cre1* strain. However, some aspects of

oxidative metabolism remain unclear, as another protein involved in electron transport (ID 123079) was upregulated in this mutant strain. By contrast, Antonieto *et al.* [23] demonstrated that, in cellulose, Δ*cre1* exhibits increased expression of these genes, in particular Mn superoxide dismutase (ID 66345) and Cytochrome P450 CYP4/CYP19/CYP26 subfamilies (ID 4517). Altogether, these results suggest that *T. reesei* has distinct mechanisms to control its oxidative metabolism in different carbon sources, representing an efficient strategy to conserve energy in response to carbon sources of varying complexity.

Another interesting finding revealed by this study was upregulation of the gene encoding the gene *ooc1* (ID 50323) by Δ*cre1* in sophorose. The *ooc1* transcript is thought to be detectable only during growth in cellulose under darkness, but not in cellulose in light or in the presence of other cellulase inducers (sophorose, lactose) [61]. Our results with the Δ*cre1* mutant strain suggest that the induction of gene expression by sophorose might correlate with cellulose in the absence of this TF, and CRE1 might be involved in signaling pathways related to cellulase induction in daylight conditions. However, Tisch and Schmoll [62] in a study using strains lacking the photoreceptors BLR1 and BLR2 as well as ENV1, showed that *cre1* is not among the targets of the light signaling machinery in *T. reesei.*

Altogether, we conclude that the complexity of the carbon source used by *T. reesei* directly influences CRE1-mediated CCR as shown in (Fig. **5**). We believe that the fungus possibly recognizes glucose derived from different carbon sources and activates the transcription factor CRE1, which binds different target genes by a mechanism not yet established. Although sophorose is a dimer derived from cellulose, we can observe that the genes repressed by CRE1 in its presence are not the same as when grown in cellulose. This occurs because the cellulose polymer also releases cellodextrins, molecules of varying length comprising two or more glucose monomers [63]. Our group previously showed that the glucose derived from these cellodextrins mainly inhibits genes related to oxidative metabolism in the initial steps of cellulose degradation [23]. However, there are common aspects of CRE1-mediated CCR in cellulose and glucose, because sophorose is one of the cellodextrins derived from the cellulose polymer. Yet, when evaluated alone, genes repressed by CRE1 in sophorose are mainly those encoding MFS permeases including maltose permeases (possible sophorose transporters) and a larger number of CAZy genes (including GH61), when compared to cellulosic growth. Although the same classes of genes appear to be repressed in both conditions, the difference is reported mainly in the target genes, confirming that CRE1 employs fine regulation to promote the appropriate adaptation of the fungus to the environment that it inhabits.

5. CONCLUSION

The present study contributes to a better understanding of the role of the TF CRE1 in the metabolic response of *T. reesei* cultivated in sophorose. Our transcriptomic analysis identified several genes whose expression is affected in a carbon source-dependent manner. The main differentially expressed genes include genes for cellulolytic enzymes,

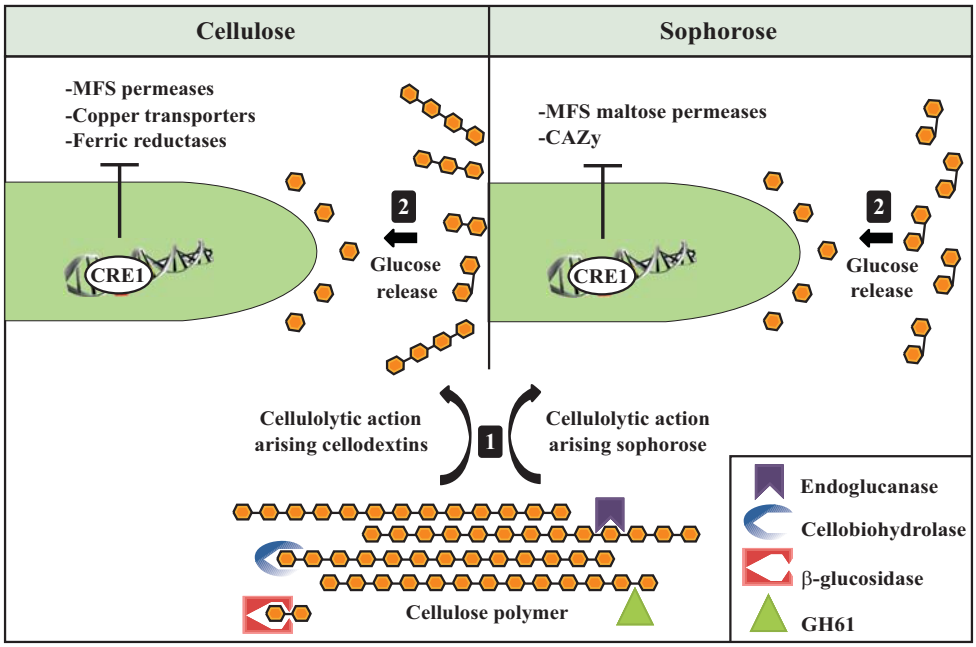

Fig. (5). Comparison of the CRE1-mediated CCR mechanism under conditions of cellulose and sophorose. Initially, the action of cellulolytic enzymes in the cellulose polymer can release cellodextrins and sophorose (1). The enzymatic hydrolysis of these oligomers releases glucose (2) which signals that the synthesis of new cellulolytic enzymes must be terminated by the fungus, since there is sufficient energy in the medium. By a mechanism not yet elucidated, the fungus can differentiate glucose arising from cellodextrins from that derived from sophorose, and different genes are suppressed in each case. In cellulose, CRE1 acts primarily on genes involved in the early stages of cellulose deconstruction, whereas in sophorose this transcription factor acts by inhibiting a greater number of genes belonging to CAZy and membrane permeases, possibly including maltose permeases that could transport sophorose.

transporters and TFs. Interestingly, most of the genes were possible indirectly regulated by CRE1. A recently study from our group demonstrated the CRE1-mediated repression of genes encoding proteins involved in the oxidative metabolism of the lignocellulosic polymer in the presence of cellulose. Yet, we also described the repression of genes related to the entry of cellulase inducers into the cell when the fungus was grown in glucose. In this work, we can observe a different profile of genes repressed by CRE1 in the presence of sophorose, reinforce the evidence for variable mechanisms of action of CRE1 in different carbon sources and demonstrating the ability of the fungus to adapt to diverse environmental conditions. These findings will contribute to understanding of the molecular mechanisms underlying the regulation of cellulolytic enzyme synthesis.

LIST OF ABBREVIATIONS

RNA-Seq	=	RNA Sequencing
CCR	=	Carbon catabolite repression
bp	=	Base pair
GB	=	Gigabase
qRT-PCR	=	Quantitative real-time PCR
GO	=	Gene Ontology
GH	=	Glycosyl hydrolase
CE	=	Carbohydrate esterase
CAZy	=	Carbohydrate-active enzyme
TF	=	Transcription factor
MFS	=	Major facilitator superfamily
ABC transporter	=	ATP-Binding Cassette transporter
FC	=	Fold change

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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DISCLOSURE

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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