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Expression pattern of cellulolytic and xylanolytic genes regulated by transcriptional factors XYR1 and CRE1 are affected by carbon source in *Trichoderma reesei*

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ABSTRACT

Trichoderma reesei is the most important fungus for the industrial production of enzymes to biomass deconstruction. Most of the genes encoding cellulases and hemicellulases are regulated by the transcription factors CRE1 and XYR1. In this work, the regulation of 22 genes of cellulases and xylanases by these transcription factors was investigated under three different carbon sources. Analysis of gene expression and enzymatic profiles of CMCase, β-glucosidase, and xylanases showed different regulation that was depended of the carbon source in both Δ*xyr1* and Δ*cre1* mutants. In the presence of glucose, the majority of genes evaluated (82%) showed increased expression levels in the Δ*cre1* mutant compared to the parental QM9414 strain. In the Δ*xyr1* mutant, it was observed that expression of cellulase and xylanase genes was reduced compared to the parental QM9414 strain, when cultured in the presence of cellulose or sophorose. Interesting, in the presence of glucose, approximately 60% of the analyzed genes had increased expression in the Δ*xyr1* mutant compared to parental strain. Furthermore, no correlation between gene expression and the number of putative binding sites of XYR1 and CRE1 to promoter region of cellulolytic and xylanolytic studied genes was observed. Therefore, these results demonstrated that the regulation of cellulase and xylanase by the transcription factors CRE1 and XYR1 is influenced by different carbon sources.

The ascomycete *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) is one of the most well-studied fungi used in biotechnology as a potential source of industrial cellulases and xylanases for biomass degradation (Saloheimo and Pakula, 2012). The cellulolytic system of this fungus consists of at least 3 different types of enzymes: exoglucanases (cellobiohydrolases EC 3.2.1.91), endoglucanases (EC 3.2.1.4), and β-glucosidases (EC 3.2.1.21), which may present under different isoforms (Saloheimo et al., 1994). Additionally, hemicellulases are also necessary for the hydrolysis of vegetal biomass. Theses enzymes act in the hydrolysis of hemicellulose, which xylan is the most abundant component, and increase the efficiency of cellulases action (Cabero et al., 2012). The fungus *T. reesei* has 5 genes that encode for xylanases, designated *xyn1* to *xyn5*, and 16 genes that encode hemicellulases (Foreman et al., 2003; Herold et al., 2013). Regulation of genes that encode cellulases and hemicellulases has been extensively studied in *T. reesei* (Schmoll and Kubicek, 2003; Stricker et al., 2008). Five transcription factors (TFs) important in this process have been previously described; positive regulators such as XYR1, ACE2, and the HAP2/3/5 complex, and negative regulators such as ACE1 and the carbon catabolic repressor CRE1 (Portnoy et al., 2011b).

XYR1 is the main transcriptional activator of genes that encode hydrolases such as *xyn1*, *xyn2*, *bxl1*, *cbh1*, *cbh2*, *egl1*, and *bgl1*. Functional XYR1-binding sequences have been described to be not only 5′-GGCTAA-3′ motifs arranged as an inverted repeat (Rauscher et al., 2006), but also as a single motif which have A or T substitutions in the 3′-proximal three bases, 5′-GGC(A/T)$_3$-3′ (Furukawa et al., 2009). Deletion of *xyr1* eliminates the induction of cellulases in the presence of cellulose and sophorose, highlighting its essential role in induction (Stricker et al., 2008). The regulation of cellulases and hemicellulases expression is also a target of carbon catabolic repression (CCR). In *T. reesei* CCR is mediated by CRE1 (Strauss et al., 1995), a Cys2His2 zinc finger protein homologous to the CreA from *Aspergillus nidulans* (Dowzer and Kelly, 1991). This

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protein has a consensus sequence at its binding site, 5′-SYGGRG-3′, that was identified in the promoters of *cbh1*, *xyn1*, *cbh2*, *egl1*, and *egl2* in *T. reesei* (Ilmen et al., 1997). It has been postulate that CCR would only occur if CRE1 bind in double active sites and this binding is mediated by specific serine phosphorylation (Portnoy et al., 2011a). An analysis of *cre1* deletion mutants demonstrated that catabolic derepression was not sufficient to increase cellulase and xylanase production, suggesting that overexpression of these enzymes depends on inducers (Nakari-Setala et al., 2009).

The sequencing of *T. reesei* genome allowed the identification of new targets for XYR1 and CRE1 (Kubicek, 2012). However, the effects of regulation of XYR1 and CRE1 on expression of cellulases or xylanases are described for a limited number of genes. These studies were restricted in the presence of only cellulose/lactose as carbon sources (Ilmen et al., 1997; Portnoy et al., 2011a). In this report, we used three different carbon sources to analyze 22 genes of cellulases and xylanases regulated by XYR1 and CRE1 in *T. reesei*. We also investigate a possible relation between the number of putative binding sites for XYR1 and CRE1 and gene expression regulation. The results demonstrated that the regulation of cellulase and xylanase by the transcription factors CRE1 and XYR1 is carbon source dependent and no correlation between gene expression and the number of putative binding sites of XYR1 and CRE1 to promoter region of 22 studied genes was observed. Our findings provide new insights into the regulation of cellulase and xylanase biosynthesis in *T. reesei* and contribute for improvement of biomass-degrading enzymes production for applications in the biofuel industry.

1. Results

1.1. In silico analysis of putative binding sites for XYR1 and CRE1 in promoter regions of cellulolytic and xylanolytic genes

In order to establish a possible relationship between the expression of cellulolytic and xylanolytic genes and the number of putative binding sites of XYR1 and CRE1, we looked for potential binding motifs in the 5′-upstream region (1.5 kbp) of 22 cellulolytic and xylanolytic genes. Among several possibilities generated by the sequence 5′-GGCWWW-3′, we compared the frequency presence of the motifs in the 1.5 kbp 5′-upstream region of the 22 XYR1-regulated genes with to the whole *T. reesei* genes. The 5′-GGCTAA-3′, 5′-GGCTAT-3′ and, 5′-GGCAAA-3′ motifs were found at 5.3-, 2.3-, and 1.6-fold higher frequencies in the XYR1-regulated genes compared to the other ORFs. In this way, we identified 78 single binding sites and 16 sites arranged as an inverted repeat sequence in the promoter region of 22 analyzed genes (Fig. 1). On the other hand, for the catabolic repressor CRE1, 31 double binding motifs were found in the promoter region of 22 analyzed genes. Among these motifs, 18 were identified to bind directly and 13 arranged as an inverted repeat (Fig. 1).

The genes that showed more putative binding sites for XYR1 were *cel7a*, *xyn3*, *cel6a*, and *cel45a* (18, 9, and 7 sites respectively) (Fig. 1). Among them, *cel7a*, *cel6a* showed increased expression in QM9414 when cellulose (Fig. 3A) and sophorose (Fig. 3B) were used as inducers and reduced expression in presence of glucose (Fig. 3C), whereas *cel45a* and *xyn3* showed reduced expression in presence of cellulose (Fig. 3A) and sophorose (Fig. 3B) and *cel45a* showed increased expression in presence of glucose (Fig. 3C). Opposite results were found for the mutant Δ*xyr1* when the same genes were analyzed (Fig. 3). Among the genes with low numbers of XYR1 motifs binding *cel74a* and *cel3a* (only one site for each gene) presented similar behavior to *cel7a*. Furthermore, for each carbon source, genes were grouped into different clusters, suggesting dependence between XYR1 and the carbon source in the regulation of some cellulolytic and xylanolytic genes (Fig. 3). For CRE1, only *cel1b* and *cel3e*, encoding for β-glucosidases, showed 6 putative binding sites. Six genes (*cel45a*, *cel3a*, *cel5b*, *xyn4*, *cel7b* and *cel12a*) did not present any double binding motif for CRE1 (Fig. 1). Analyzing the mutant Δ*cre1*, most genes with the high number of motifs for CRE1 binding showed an increase of expression on cellulose, sophorose and glucose (Fig. 3). On the other hand, genes with no binding site for this factor were also up regulated in the three carbon sources (Fig. 3). Interestingly, even in the Δ*xyr1* mutant, *cel1b* and *cel3e* also showed high expression in cellulose, sophorose, and glucose compared with QM9414 (Fig. 3), suggesting cooperation between XYR1 and CRE1 in the regulation of these genes. Together, *in silico* analysis results provide no evidence of a correlation between the expression of cellulolytic and xylanolytic genes and the number of putative binding sites for both XYR1 and CRE1.

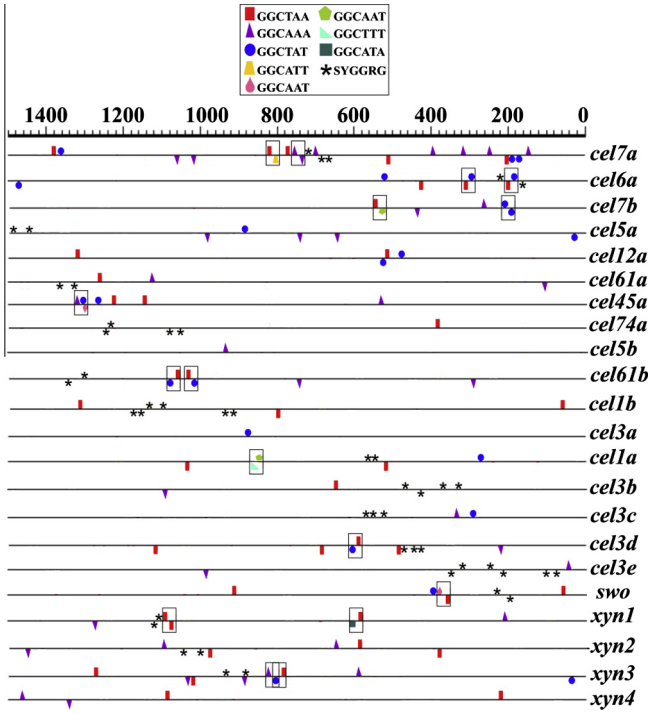

Fig. 1. Schematic representation of putative binding sites of XYR1 (5′-GGCWWW-3′) and CRE1 (5′-SYGGRG-3′) binding in the 1.5-kbp upstream region of cellulolytic and xylanolytic genes. The position of the motifs is relative to the translation initiation codon (ATG). The symbols above and below the lines indicate binding motifs in the sense and nonsense DNA strand respectively. The binding motifs for CRE1 are represented by inverted and direct repeats. XYR1 inverted repeats sites are highlighted (rectangle) and simple sites are indicated at the legend. W = A/T, S = C/G, Y = C/T e R = A/G, according to IUPAC norms.

1.2. Analysis of the influence of carbon source on regulation of cellulolytic and xylanolytic gene expression by XYR1 and CRE1

To guarantee comparisons between strains, we first examined the growth pattern of strains on cellulose and glucose. As shown in Fig. 2A and B, only Δ*cre1* showed a discrete difference ($P > 0.05$) in growth when compare to the parental QM9414. No differences was observed in glucose consuming at 24 and 48 h and at the end of experiment about 25% of glucose was still available for strains, excluding the possibility of stress by nutrient exhaustion (Fig. 2C). Furthermore, the pH behavior was the same for all strains during glucose cultivation, ranged from 4.85 ± 0.12 at 0 h to 3.88 ± 0.075 at 48 h of cultivations.