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Dissertation

Impact of chromatin remodeling on the expression of Xyr1 regulon genes in *Trichoderma reesei*

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Table of Contents

Kurzfassung

Der Ascomycet *Trichoderma reesei* wird für die Produktion von Zellulasen und Hemizellulasen im industriellen Bereich verwendet. Die Expression der Gene, die diese Enzyme kodieren, wurde bisher hauptsächlich auf Ebene der Transkriptionsregulation durch regulierende Proteine erforscht, insbesondere die Transaktivatoren Xyrl (Xylanase Regulator 1) und der Repressor Cre1(Carbon Katabolit Repressor 1), deren Zusammenspiel hauptsächlich die Expression dieser Enzyme reguliert. Den Auswirkungen von Chromatin-Remodellierung, d.h. der dynamischen Modifikation der Chromatin-Architektur um den Zugang der regulatorischen Proteine an die genomische DNA zu ermöglichen, auf die Genexpression in *T. reesei* wurde bisher kaum Aufmerksamkeit geschenkt.

Einer der wichtigsten Stämme vom *T. reesei* ist RUT-C30 ein hyperzellulolytischer Mutant, auf dem die meisten Stämme für die industrielle Herstellung von Enzymen, insbesondere Zellulasen, basieren. Ein wichtiges genetisches Merkmal von RUT-C30 ist eine teilweise Deletion des Gens *cre1*, durch die der Stamm partiell von Kohlenstoff Katabolit Repression (KKR) freigesetzt wird. Diese Deletion wird als Hauptgrund für den herausragenden Phänotyp dieses Stammes angesehen. Es ist jedoch noch unklar, was genau die Zellulaseproduktion in RUT-C30 verbessert.

Diese Forschungsarbeit bringt neue Erkenntnisse über die Genexpression von Zellulasen und Hemizellulasen bezogen auf den Chromatin-Zugang von Cre1 und Xyr1. Sie erforscht weiters die Verkürzung von Cre1 in RUT-C30 als eine zusätzliche verstärkende Eigenschaft dieses Stammes, die über einfache Befreiung von KKR hinausgeht. Die hier vorgestellten Ergebnisse weisen darauf hin, dass die Verkürzung von Cre1 zu Cre1-96 in Rut-C30 einen positiven regulatorischen Einfluss auf die Expression hat. Sie wirkt möglicherweise nicht nur in direkter Weise auf den Promotor von Zielgenen, sondern trägt durch die Regulierung eines Gens, das ein Protein zur Chromatin-Remodellierung kodiert, auch indirekt zu einem offeneren Chromatin-Status bei. Andererseits beeinflusst Xyr1 auch die Chromatin-Verpackung. Chromatin-Zugänglichkeit

Echtzeit-PCR in Kombination mit Transkriptanalyse ergab, dass Xyr1 für die volle Induktion von Zellulase-kodierenden Genen durch Sophorose erforderlich ist und, dass höhere Genexpressionen mit Chromatin-Öffnung einhergehen. Darüber hinaus offenbart RNA-seq Analyse, dass Gen-Produkte, die an Histon-Acetylierung und ATP-abhängiger Chromatin-Remodellierung beteiligt sind, auch Chromatin Öffnung während Sophorose-Induktion beeinflussen. Schließlich wurde der Promotor von *xyr1* auch als Ziel von Chromatin-Remodellierung enthüllt. Chromatin-Umlagerung erfolgt im *xyr1* Promotor während der Induktion durch Sophorose. Diese Umlagerung erfolgt vor der Aktivierung von Zellulase Genexpression und die Zugänglichkeit des Promoters in einem Cre1-freien Hintergrund ist insgesamt höher, egal welche Kohlenstoffquelle vorhanden ist. Zusammenfassend ist die Regulierung der Genexpression von Zellulasen und Hemizellulasen in *T. reesei* nicht nur auf die Wirkung von Transkriptionsfaktoren beschränkt, sondern steht offensichtlich auch in Beziehung zu Änderungen in der Chromatin-Verpackung.

Abstract

The ascomycete *Trichoderma reesei* is used for the production of plant cell wall-degrading enzymes (PCWDEs) in industrial scale. The expression of the PCWDE-encoding genes has been so far primarily investigated on the level of transcriptional regulation by regulatory proteins, especially the transactivator Xyr1 and the repressor Cre1. The interplay between these two transcription factors mainly regulates the expression of PCWDEs. Otherwise, the impact of chromatin remodeling, *i.e.* the dynamic modification of chromatin architecture to allow the regulatory proteins access to genomic DNA, on gene expression in *T. reesei* has received hardly any attention so far. One of the most important *T. reesei* strains, RUT-C30, is a hypercellulolytic mutant that became the ancestor of most industry strains used in the production of enzymes, in particular cellulases. One important genetic trait of RUT-C30 is a partial deletion of the gene *cre1*, which releases the strain from carbon catabolite repression (CCR). This deletion has been considered the main reason for the outstanding phenotype the strain presents. However, it is still unclear, what exactly enhances cellulase production in RUT-C30.

This thesis presents new insights on the regulatory mechanism behind gene expression of PCWDEs, especially concerning the role of Cre1 and Xyr1 in chromatin access. It also explores the truncation of Cre1 in RUT-C30 as an additional enhancing characteristic of this strain that goes beyond just simple CCR release. The results presented here point towards the fact that the truncated form of Cre1 of RUT-C30, Cre1-96, exerts a positive regulatory influence on the expression. It possibly acts in a direct manner on the promoter of target genes, but also contributes indirectly to a more open chromatin status by regulating a gene encoding a possible chromatin-remodeling protein. On the other hand, Xyr1 also influences the chromatin packing. Chromatin accessibility real-time PCR combined with transcript analysis showed that Xyr1 is required for the full induction of cellulase-encoding genes by sophorose and that higher gene expression overlaps with chromatin opening. Additionally, RNA-seq analysis revealed that gene products involved in histone

acetylation and ATP-dependent chromatin remodeling may also influence chromatin opening during sophorose induction. Finally, the promoter region of *xyr1* was also identified as a target of chromatin remodeling. Chromatin rearrangement occurs in the *xyr1* promoter during induction by sophorose and it takes place prior to activation of cellulase gene expression. Also, *xyr1* promoter accessibility is overall higher in a *cre1*-truncated background, no matter which carbon source is present. To sum up, the regulation of PCWDEs gene expression in *T. reesei* is not only restricted to the action of transcription factors, but is clearly related to changes in the chromatin packaging.

Introduction

Expression of plant cell wall-degrading enzymes in *Trichoderma reesei*: Cre1 and Xyr1 roleplay.

Lignocellulose is the main component of plant biomass and thus the most abundant polysaccharide present on earth. Its intricately polymeric structure is basically composed of cellulose, hemicelluloses, pectin, and polyphenol lignin. Together with plant cell wall–associated enzymes, structural proteins, and proteoglycans, lignocellulose confers rigidity and durability to the plant cell walls [1]. Degradation of plant biomass is a complex process, which requires the synergistic action of the extracellular enzymes produced by microorganisms including bacteria and fungi. Filamentous fungi are among the most efficient degraders and are the main source of commercial plant cell wall-degrading enzymes (PCWDEs) used for lignocellulose breakdown. The filamentous ascomycete *Trichoderma reesei* (*Hypocrea Jecorina*) is the most commonly used organism for commercial production of cellulases [2; 3].

Isolated during the Second World War, QM6a, the wild-type *T. reesei* strain, was soon recognized as a valuable source for research due to its high capacity for cellulases production [4]. Since then QM6a has been used in strain development programs in order to isolated mutants and/or engineered derivatives to achieve higher enzyme production for employment in the pulp and paper, food and feed, and the textile industries [5-7], as well as in the production of biofuels [8]. One of QM6a derivative mutants, the hypercellulolytic strain RUT-C30 was obtained through three steps. First, mutagenesis by ultraviolet (UV) light and screening for the release from carbon catabolite repression (CCR). Second, mutagenesis by N-nitroguanidine, followed by another round of UV mutagenesis and screening for high cellulase activity [9-11]. RUT-C30 outperforms QM6a by achieving higher levels of protein secretion (up to 20 mg of extracellular protein per ml) and higher cellulolytic activity (15-20 times more) [12; 13]. Due to its outstanding performance RUT-C30 became one of the most widely employed strains of filamentous fungi for the production of

cellulolytic enzymes and recombinant proteins, and thus for academic research [14].

Physiological and genetic changes have been reported in RUT-C30 that contribute to the high protein secretion and enhanced cellulase gene expression. Bigger endoplasmic reticulum in comparison to QM6a during cellulose induction, and higher mycelial protein content, have been observed as physiological changes associated with the secretory enhancement of RUT-C30 [15; 16]. Several genetic changes in the strain have also been identified in RUT-C30. Major changes include an overall increase of the genome size from 32.5 to 34.7 Mbp [17], rearrangements between chromosomes carrying genes encoding cellulolytic enzymes [18], the lack of more than 100 kb of genomic DNA present in QM6a (encompassing 15 small insertions and deletions and 18 larger deletions), and the presence of 223 single nucleotide variants affecting cellular processes involved in metabolism, nuclear transport, mRNA stability, sugar transport and transcription [19]. One of the deletions is a 83 kb region located in scaffold 15, which encodes 29 genes associated with primary metabolism, transporter proteins, extracellular enzymes and proteins associated with cellular detoxification [20; 21]. Another important property, which is considered the genetic basis of the the release from carbon catabolite repression (CCR) exhibited by RUT-C30, is the lack of the a version of the Carbon catabolite repressor 1 (Cre1, [22]). RUT-C30 bears a *cre1* sequence that would encode for only one of the two zinc finger regions that compose the DNA-binding domain of Cre1, resulting in a truncated protein with 96 amino acid residuals (Cre1-96) instead of 402 of the native Cre1 [23].

CCR stands for a global control system present in various microorganisms, which allows quick adaptation to a preferred (rapidly metabolisable) carbon and energy source. In *T. reesei* CCR can be achieved through inhibition of synthesis of PCWDEs, favoring the uptake and consumption of easily metabolized carbon sources such as glucose and sucrose [24]. Cre1 mediates CCR in *T. reesei* regulating both cellulose and xylan utilization, and downregulating the expression of lignocellulolytic genes [25]. The repressor is a C_2H_2-type zinc finger protein with the DNA-binding

consensus sequence 5′-SYGGRG-3′ [22], and is homologous to CreA from *Aspergillus* species [26-28] and MIG1 from *Saccharomyces cerevisiae* [29]. Cre1 regulates transcription of genes in a double-lock manner. It can directly inhibit the expression of several genes encoding for PCWDEs by binding to tandem and inverted repeats in their upstream regulatory regions (URRs) [30; 31]. Additionally, in the presence of D-glucose it represses the transcription of the Xylanase regulator 1 (Xyr1), the main transactivator of PCWDEs expression [32; 33].

On the one hand, Cre1 works as the main negative regulator of PCWDEs expression and its deletion results in derepression of cellulase and hemicellulase production in glucose-based medium [34]. On the other hand, deletion of *cre1* does not induce the expression of lignocellulolytic genes in the absence of an inducing substance, and here Xyr1 comes into play. Xyr1 is a Gal4-like Zn_2Cys_6 binuclear cluster protein essential for the expression of xylanases, cellulases and xylose metabolism-related genes [32]. It is homologous to XlnR/Xyr1/Xlr1 from *Aspergillus* species, *Fusarium graminearum*, *Neurospora crassa* and *Magnaporthe oryzae* [28; 35-39], and usually interacts with 5'-GGC(W)$_3$-3'-motifs arranged as inverted repeats in promoter regions of its target genes [40]. Within the Xyr1 regulon the activator coordinates the expression signals from various inducers (D-xylose, xylobiose, and sophorose) and regulates all modes of gene expression (basal, derepressed, and induced) of the major cellulase and hemicellulase encoding genes [32]. Interestingly, the induction of the genes coding for the main cellulases (*i.e. cbh1* and *cbh2*) is directly associated with the extent of induction/repression of *xyr1*, which is not observed for the other target genes of Xyr1 [41]. In most carbon sources *xyr1* itself is usually expressed at a low level but it can be induced by sophorose, which itself is formed by transglycosylation [33; 41].

Chromatin status and gene expression.

The events of gene expression in eukaryotic systems are regulated by constantly changing cohorts of site-specific DNA-binding proteins that direct cell-selective transcriptional

programs towards adaptive responses to specific environmental signals. These factors interact with *cis* DNA regulatory elements to control the activity states of target promoters. On the one hand, this simple site-specific recognition of DNA elements by regulatory proteins may explain the central mechanism of promoter-specific regulation in bacterial systems. However, in eukaryotes, given the larger sizes and the complexity of their genomes, additional layers of regulation become visible when unraveling the regulatory programs. The organization of their genomes into DNA-protein complexes called chromatin is not only a mechanism for packing the entire genetic information into the confines of the nucleus, but also regulates the accessibility of DNA for transcription, recombination, DNA repair, and replication [42]. The chromatin structure is dynamic and provides considerably restricted access of transcription factors (TFs) to regulatory sites in a highly condition-specific manner [43]. This variable access to regulatory elements plays a key role in different expression profiles.

Transcription factors and chromatin access.

Chromatin basically consists of the nucleosome core particle, which is composed of 147 base pairs of DNA wrapped 1.65 times around an octamer containing two copies each of the core basic histones H2A, H2B, H3, and H4 [44]. Nucleosomes are assembled into long, linear arrays in which each nucleosome is connected by 10–70 bp of linker DNA, with the length varying between species and cell types [43]. Several mechanisms regulate the dynamics of assembly, positioning, and stability of nucleosomes. One mechanism is the post-translational modification of histones, which can modulate chromatin folding [45; 46] and guide the binding of regulatory proteins [47; 48] resulting in altered activity states for both promoters and regulatory sequences. Secondly, non-allelic histone variants, such as H2A.Z or H3.3, can also be incorporated into nucleosomes, and these variant nucleosomes can have altered stability and/or present novel opportunities for post-translational modifications [42; 49]. Thirdly, several chromatin-remodeling enzymes use the energy

of ATP hydrolysis to catalyze the remodeling of chromatin by re-positioning, evicting, or altering the composition of nucleosomes [50].

During transcription, occurs the coordinated recruitment of several factors that work to facilitate the recruitment, passage and release of RNA polymerase II (Pol II) [51]. Transcription begins with the binding of sequence-specific activators to *cis*-regulatory sequence DNA elements, followed by recruitment of general TFs and assembly of the pre-initiation complex (PIC) [52; 53]. After initiation, there is a transition to productive elongation and finally, termination [54; 55]. Chromatin dynamic structure plays an important role during in this whole process. In the promoter the nucleosomes are repositioned or removed to generate nucleosome-depleted regions (NDR), hence exposing *cis*-regulatory sequences, relieving the physical barrier that they pose to the transcribing RNA polymerase. Nucleosomes are reassembled and repositioned in the wake of polymerase passage [56].

Besides activating/repressing transcription by interacting with other components of the transcriptional machinery, TFs also play an important role in regulating nucleosome occupancy at promoters. TFs may interact with the cellular enzymes that act on chromatin to either demarcate elements for action or silence these elements in a given cellular context. Examples of such interactions include the glucocorticoid receptor (GR), which through the N-terminal transactivation domain directly recruits the SWI-SNF remodeling complex to target promoters during glucocorticoid-dependent gene activation in both yeast and mammalian cells [57]. Yeast transcriptional activator INO2, necessary for expression of phospholipid biosynthetic genes in *Saccharomyces cerevisiae*, recruits both SWI/SNF and INO80 remodeling complexes during *INO1* derepression [58].

Histone modifying enzymes such as histone acetyltransferases (HATs) and histone deacetylases (HDACs) may also interact with specific DNA-binding activator and repressor proteins, respectively. Histone hyperacetylation is generally associated with chromatin

decondensation and increased transcriptional activity, whereas histone hypoacetylation contributes to chromatin condensation and transcriptional repression [59]. As an example, the human CREB-binding protein (CBP) can be recruited by STAT1 and STAT2 transactivation domains and activate gene expression via HAT activity [60]. Conversely, HDACs mediate gene repression by being recruited by TFs, including p53, Rb, YY1, NF-κB, Sp1, and Sp3, to numerous target promoters [61]

Additionally, DNA methylation of cytosine at CpG dinucleotides also participates in promoter function. In eukaryotes the hypermethylation of CpG islands (CGIs) is associated with transcriptional repression either through methyl cytosine binding proteins (MBPs) recruiting HDACs [62; 63] or through blocking transcription factor binding in some special cases [64-66]. On the other hand, methylated cytosine can also attract transactivators. Methylation of the *cis* CRE sequence, for example, enhances the DNA binding of C/EBPα, which in turn activates a set of promoters specific for adipocyte differentiation [67; 68].

Since *T. reesei* is a eukaryote, it is reasonable to imagine that the main TFs controlling PCWDEs expression, Cre1 and Xyr1, also have some influence on chromatin packing. Nevertheless, data pointing to such a regulatory role is still scarce and the mechanism of the phenomenon is poorly understood.

Aims

The aims of this thesis were to obtain new insights into the regulatory mechanism behind gene expression of PCWDEs concerning chromatin access and the influence of Cre1 and Xyr1 in this process. The first aim was to investigate to what extent the absence of a complete Cre1 contributes to the cellulase hyper-producing phenotype of Rut-C30. A special focus was to study the role of the remaining truncated version of Cre1 with regard to the transcriptional regulation of target gene expression, the ability of DNA binding, and the influence on the chromatin structure. The second aim was to learn if the antagonist of Cre1, the transactivator Xyr1, is also involved in chromatin remodeling, and if this happens in a condition (inducing/repressing carbon source)-dependent way. Thirdly, the *xyr1* promoter architecture was investigated in regards to being a possible target for chromatin remodeling and the impact of this would have on the cellulase expression in a comparative study including RUT-C30 and the wild-type strain.

Conclusions

Since the isolation of QM6a, unraveling the mechanisms behind the expression PCWDEs has been a strong research topic in order to understand the biology of *T. reesei*. Nevertheless, while several efforts have been made to learn about the key TFs involved in the regulation of *T. reesei*'s PCWDE-encoding genes [25; 69-72] (and reviewed in [2; 73]), so far little is known about the impact of the chromatin status on their gene expression. Only for Cre1 was already suggested that this TF might have influence chromatin remodeling. By analyzing loss-of-function strains for Cre1, former reports point towards this transactivator as an important factor in influencing nucleosome positioning in the upstream regulatory regions (URR) of *cbh1* and *cbh2* during repressing conditions [74; 75].

The first publication within this thesis brings evidences that the deletion in the *cre1* locus in RUT-C30 not only confers the strain partial release from CCR, but the truncated version of Cre1 (namely Cre1-96) should be considered as a discrete transcription factor with different properties from Cre1 [76]. In contrast to Cre1, Cre1-96 seems to act on target URRs in a carbohydrate-independent manner and contributes to chromatin opening. In addition, this truncated Cre1 may indirectly contribute to chromatin remodeling by regulating the expression of *htf1,* a putative helicase that might take part in an ATP-dependent chromatin remodeling complex [76]. Supporting this hypothesis, there is evidence that *hft1* (also referred as *snf2*) is a target under control of Cre1 [25].

Furthermore, the major regulator of cellulases and xylanases expression, Xyr1 was also shown to contribute to chromatin remodeling during PCWDEs expression by partially coordinating the sophorose-inducing signal [77]. Decreased cellulase gene expression in the absence of Xyr1 was observed to go along with denser chromatin, yet a minor sophorose-mediated chromatin oppening was measured in the Δ*xyr1* strain. Moreover, 15 gene targets possibly involved in chromating remodeling proccesses were indentified by RNA-seq during sophorose-induction, 14 of which were

expressed independently of Xyr1 and may be part of ATP-dependent chromatin remodeling complexes and/or proteins involved in histone acetylation. Notably, the gene with transcript ID 73708, encoding a putative heterochromatin-associated protein, was downregulated on sophorose compared to D-glucose and upregulated in the absence of Xyr1 [77].

Finally, the URR of *xyr1* was shown to be a target for chromatin remodeling and the investigation of chromatin accessibility of the *xyr1*, *cbh1*, and *cbh2* promoters revealed that during early induction chromatin rearrangement targets primary the *xyr1* promoter [78]. These results corroborates with the observation that the induction of *cbh1* and *cbh2* is directly associated with the extent of induction of *xyr1* expression, which is not observed for the other target genes of Xyr1 [41]. Additionally, *in vivo* footprinting analysis detected an upstream regulatory region in the *xyr1* promoter to be an important target of chromatin opening, probably involving regulation by Cre1 interaction and the protein complex HAP2/3/5, besides a possible auto-regulatory Xyr1-binding site [78].

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RESEARCH ARTICLE Open Access

A truncated form of the Carbon catabolite repressor 1 increases cellulase production in *Trichoderma reesei*

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Abstract

Background: Rut-C30 is a cellulase-hyperproducing *Trichoderma reesei* strain and, consequently, became the ancestor of most industry strains used in the production of plant cell wall-degrading enzymes, in particular cellulases. Due to three rounds of undirected mutagenesis its genetic background differs from the wild-type QM6a in many ways, of which two are the lack of a 83 kb large sequence in scaffold 15 and the partial lack of the gene encoding the Carbon catabolite repressor 1 (CREI). However, it is still unclear, what exactly enhances cellulase production in Rut-C30.

Results: The investigation of the expression of two genes encoding cellulases (*cbh1* and *cbh2*) and the gene encoding their main transactivator (*xyr1*) revealed that the presence of the truncated form of CREI (CREI-96) contributes more to the Rut-C30 phenotype than a general loss of CREI-mediated carbon catabolite repression (*cre1* deletion strain) or the deletion of 29 genes encoded in the scaffold 15 (83 kb deletion strain). We found that the remaining *cre1* in Rut-C30 (*cre1-96*) is transcribed into mRNA, that its putative gene product (Cre1-96) is still able to bind DNA, and that the CREI-binding sites in the upstream regulatory regions of the chosen CREI-target genes are still protected in Rut-C30. As it was previously reported that CREI acts on the nucleosome positioning, we also analyzed chromatin accessibility of the core promoters of CREI-target genes and found them open even on D-glucose in the presence of CREI-96.

Conclusions: The lack of the full version of CREI in Rut-C30 corresponds with a partial release from carbon catabolite repression but is not completely explained by the lack of CREI. In contrast, the truncated CREI-96 of Rut-C30 exerts a positive regulatory influence on the expression of target genes. Mechanistically this might be explained at least partially by a CREI-96-mediated opening of chromatin.

Keywords: *Trichoderma reesei*, *Hypocrea jecorina*, Rut-C30, Cellulases, Carbon catabolite repressor 1, Chromatin

Background

The filamentous ascomycete *Trichoderma reesei* is a saprophyte known for its ability to efficiently degrade biomass material by plant cell wall (PCW)-degrading enzymes. A genome-wide analysis identified 10 cellulolytic and 16 hemicellulolytic enzyme-encoding genes in *T. reesei* [1], of which the two most prominent cellulose-degrading enzymes are the cellobiohydrolases CBHI and CBHII (EC 3.2.1.91) [2]. However, the defining feature of this fungus is the exceedingly high amount of secreted enzymes that provoked its industrial exploitation for their production. Next to the pulp and paper industry [3], the food and feed industry [4], and the textile industry [5], these enzymes are applied in the production of biofuels [4]. With regards to cellulosic ethanol, the production costs of the needed enzymes massively influence the price and the competitiveness of the end-product. As a result their efficient expression remains an important topic of research.

The ancestor of most current industry strains is Rut-C30 [6,7]. It was derived from the *T. reesei* wild-type isolate QM6a through three rounds of mutagenesis (ultraviolet (UV) light and N-nitroguanidine) followed by a screening

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for the release from carbon catabolite repression (CCR) and high cellulase activity [8-10]. Since then the study of the specific physiological and genetic changes in Rut-C30 has been of interest [7,11] and a number of properties have been identified, for example, a 83 kb large region located in scaffold 15, which encodes 29 genes is lacking in Rut-C30 [12,13]. Another important property of Rut-C30 is the lack of the full version of the Carbon catabolite repressor 1 (CREI, [14]) having left a *cre1* sequence that would only encode for one of the two zinc finger regions of CREI (96 aa long) [15]. The native CREI is a C_2H_2-type zinc finger protein with the consensus sequence 5′-SYGGRG-3′ [14], and is orthologous to CreA from *Aspergillus* sp. [16-18] and MIG1 from *Saccharomyces cerevisiae* [19].

In *T. reesei* CREI is known to act on a number of regulatory levels. Firstly, it directly represses transcription of several genes encoding for PCW-degrading enzymes, for example the *cbh1* gene [20], by binding to tandem and inverted repeats in their upstream regulatory regions (URRs) [20,21]. Secondly, in the presence of D-glucose it represses the expression of the main transactivator of PCW-degrading enzyme expression, the Xylanase regulator 1 (XYRI) [22,23]. XYRI is a Gal4-like Zn2Cys6 binuclear cluster protein, of which the expression can be induced by sophorose [6] or as above-mentioned, repressed by D-glucose. Notably, the extent of induction of *xyr1* gene expression directly correlates with the induction of *cbh1* and *cbh2* gene expression, which is not the case for other genes in the XYRI regulon [6]. As a third regulatory level, CREI has been reported to play an essential role in correct nucleosome positioning, for example in the promoters of the *cbh1* and *cbh2* genes [24,25]. Altogether, CREI plays a major role in the regulation of 250 genes, both in a repressing and inducing way [26].

In this study we investigated to which extent the two mentioned striking genetic properties of Rut-C30, namely the lack of the 83 kb in scaffold 15 and the absence of a full CREI contribute to its cellulase hyper-producing phenotype. A special focus was studying the role of the remaining truncated version of CREI with regard to the transcriptional regulation of target gene expression, the ability of DNA binding, and the influence on the chromatin structure. The PCW-degrading enzyme-encoding genes *cbh1* and *cbh2*, as well as *xyr1* as the gene encoding their transactivator, were chosen as the CREI-target genes to be analyzed.

Results

The expression profile of *cbh1*, *cbh2*, and *xyr1* in QM6a-CREI$_{96}$ is closest to Rut-C30

In order to find out which genetic properties of Rut-C30 contribute to its cellulase hyper-producing phenotype we compared its expression profile to three transgenic strains. Two of them bear Rut-C30-related mutations, namely Δscaff15, which is a QM6a-derived strain with an 83 kb deletion in scaffold 15, and QM6a-CREI$_{96}$, which bears the truncated CREI version like Rut-C30 does. We also included a QM6a-derived *cre1* deletion strain because the phenotype of Rut-C30 is sometimes associated with the lack of CREI. We investigated expression levels of *cbh1*, *cbh2*, and *xyr1* under sophorose-inducing conditions. Interestingly, the transcript levels of all three genes were most similar in QM6a-CREI$_{96}$ compared to Rut-C30, while in the case of the other two strains (Δ*cre1* and Δscaff15) considerably lower levels were detected (Figure 1a, b, c). This result raised the consideration that regulatory molecular mechanisms are related to the truncated version of *cre1* remaining in the Rut-C30 genome.

CREI sites in upstream regulatory regions of target genes are strongly protected from DNA methylation in Rut-C30

All above-mentioned analyzed genes (*cbh1*, *cbh2*, and *xyr1*) are under the regulatory influence of CREI [20,22,25]. To learn if the protection patterns against DNA methylation of the URRs bearing CREI sites of these genes differ between the wild-type QM6a and Rut-C30, we performed *in vivo*/*in vitro* footprinting analyses. URRs of all three genes were investigated after both strains were pre-grown on glycerol and then incubated on D-glucose for 3 hours followed by dimethyl sulphate (DMS)-induced *in vivo* methylation. The footprinting pattern obtained for all genes showed the same or even stronger DNA occupancy in Rut-C30 compared to QM6a (Figure 2a, b, c). This finding prompted us to analyze if the truncated *cre1* (*cre1-96*) in Rut-C30 is transcribed. Thus, after pre-growth both strains were transferred to media containing D-glucose as a repressing condition, sophorose as an inducing condition, and no carbon source as a control condition respectively, and incubated for 3 hours. While we detected a low basal transcript level (originating from the native *cre1* gene) in the wild-type, we found increased levels in Rut-C30, whereupon the increase was more pronounced on D-glucose and sophorose than without carbon source (Figure 3a). Since the *cre1-96* mRNA could be detected in high amounts in Rut-C30 regardless of the applied condition (D-glucose or sophorose), we again performed *in vivo/in vitro* footprinting to investigate if the DNA protection pattern changes condition-dependent. We analyzed the same URRs of *cbh1*, *cbh2*, and *xyr1* genes comparing the application of D-glucose and sophorose. As can be inferred from Figure 3b, c, d no condition-specific differences could be detected even though strong DNA occupancy at the CREI sites was observed. This fits the expression results and suggests that CREI-96 may no longer act in a D-glucose specific manner.

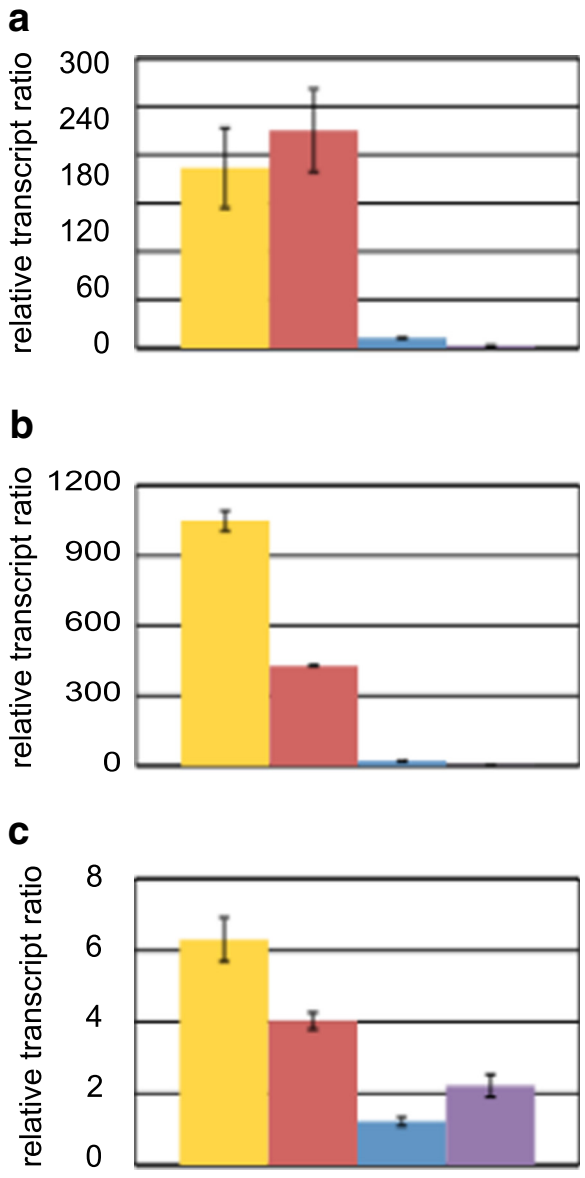


Figure 1 Transcript analysis in *Trichoderma reesei* strains bearing Rut-C30-related mutations. Rut-C30 (yellow bars), QM6a-CREI$_{96}$ (red bars), the Δ*cre1*-strain (blue bars), and the Δscaff15-strain (purple bars) were pre-grown on glycerol and then transferred to medium supplemented with sophorose and incubated for 3 hours. The transcript level analysis of the *cbh1* **(a)**, *cbh2* **(b)**, and *xyr1* **(c)** genes was performed by qPCR using *sar1* and *act* genes for data normalization. Levels refer to the wild-type strain QM6a incubated without carbon source. The values are means from measurements and biological experiments (cultivations) performed in triplicate. Error bars indicate standard deviations.

The truncated CREI-96 protein of Rut-C30 can bind to DNA *in vitro*

The results described above raise the question of whether the putative CREI-96 protein is still able to bind to DNA, especially because it lacks one of the two zinc fingers. To answer this question, we performed an electrophoretic mobility shift assay (EMSA) using the URR of *cbh1* covering three functional CREI-binding sites as a probe (Figure 4a). The applied CREI-96 protein was heterologously expressed as GST-fusion protein and thrombin-cleaved before usage. The assay yielded two bands (Figure 4b), which represent most likely the binding of one or more CREI-96 proteins to the sites of the probe. Similar results were observed before reporting that shorter versions of CREI can still bind target DNA sequences yielding more than one band [28]. However, both complexes are specifically formed because the bands were diminished by adding a cold competitor, while they remained unchanged by adding a specifically mutated competitor (Figure 4b). Altogether, this supports the working hypothesis that the truncated CREI-96, which is putatively formed in Rut-C30, is still able to bind its DNA target sequences. It should be noted that in filamentous fungi other regulatory proteins are known that are also able to bind their DNA with a single zinc finger, such as the *Aspergillus nidulans* AreA [29].

Higher gene expression corresponds with more open chromatin in QM6a-CREI$_{96}$

As we had strong indication that the truncated version of CREI-96 is present in Rut-C30 and still is able to bind the URRs of its target genes, we aimed to get more insights on its potential regulatory role. Consequently, QM6a-CREI$_{96}$ and the *cre1* deletion strain were grown on D-glucose and samples were drawn after 36, 39, 42, and 45 hours of cultivation. Because CREI was reported to be involved in nucleosome positioning within the *cbh1* and the *cbh2* promoter of *T. reesei* [24,25], we investigated the chromatin packaging by applying chromatin accessibility real-time PCR (CHART-PCR) analysis of the core promoter regions of the *cbh1*, *cbh2*, and *xyr1* genes. Second, we investigated the expression of these genes by RT-qPCR to see if there was a correlation of transcript levels with the chromatin accessibility. We could detect a higher expression of *cbh1* and *cbh2* genes in QM6a-CREI$_{96}$ compared to the Δ*cre1*-strain, which corresponded with a more open chromatin in the core promoters of the two genes in QM6a-CREI$_{96}$ (Figure 5a, b). The higher transcript levels of cellulase-encoding genes in QM6a-CREI$_{96}$ were reflected by increased enzyme activity (45.8 ± 1.5 U/mg dry weight;) measured after 45 hours of cultivation on D-glucose compared to the Δ*cre1*-strain (29.1 ± 1.8 U/mg dry weight). Interestingly, we found that in QM6a-CREI$_{96}$ the gene expression increased with a simultaneous opening of chromatin, in particular in *xyr1* and *cbh1* (Figure 5a). On the other hand, we could not observe a correlation of gene expression and chromatin accessibility in the *cre1* deletion strain (Figure 5b).

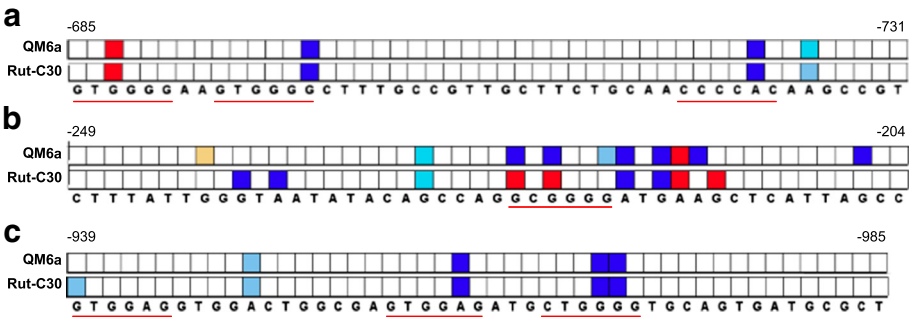


Figure 2 *In vivo* and *in vitro* footprinting analyses of URRs of CREI-target genes. The *Trichoderma reesei* wild-type strain QM6a and Rut-C30 were pre-grown on glycerol and then incubated on D-glucose for 3 hours followed by DMS-induced *in vivo* methylation. An URR bearing functional CREI sites (underlined in red) of the *cbh1* **(a)**, *cbh2* **(b)**, and *xyr1* **(c)** genes was investigated, and methylated, naked DNA was used as the reference. Numbers indicate the position of the base upstream from ATG. Analysis of data and visualization was performed using ivFAST (*in vivo* footprinting analysis software tool) [27]. Only signals that are statistically different are considered. Protected bases are highlighted in red shades and hypersensitive bases are highlighted in blue shades. The three colour intensities each correspond to stronger differences between compared conditions; increasing colour intensity means more than 1.4-, 1.6-, and 1.8-fold difference in *cbh1* and *cbh2* **(a, b)**, and more than 2.4-, 2.6-, and 2.8-fold difference in *xyr1* **(c)**.

To discover what this scenario looks like under inducing conditions, we applied the same experimental strategy on the two strains after pre-growth followed by incubation on sophorose for 30, 90, and 180 minutes. For *cbh1* and *cbh2* we observed in both strains an increase in transcript levels over time, which did not correlate in either of the two strains with a simultaneous opening of chromatin (Figure 6a, b). In the *xyr1* gene, transcript levels did not increase however, the chromatin became more compact over time (Figure 6a, b). Comparing the chromatin status of the two strains, the chromatin was slightly more accessible in QM6a-CREI$_{96}$. (Figure 6a, b).

CREI-96 upregulates the expression of a helicase-like transcription factor

Due to the results obtained from the CHART-PCR analyses, we assumed that the truncated CREI-96 was involved in chromatin remodelling in both a direct and indirect

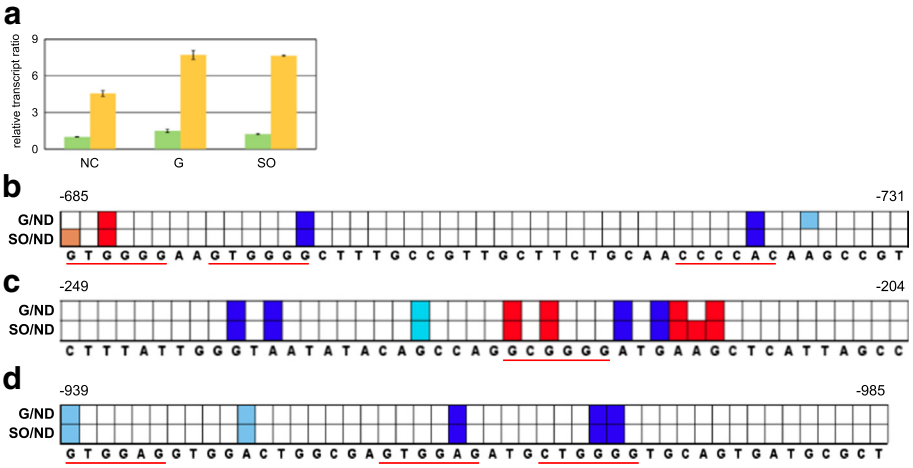


Figure 3 Analysis of the abundance of CREI-96 in Rut-C30. (a) The *Trichoderma reesei* wild-type strain QM6a (green bars) and Rut-C30 (yellow bars) were pre-grown on glycerol and then transferred to media supplemented with D-glucose (G), sophorose (SO) or without carbon source (NC), respectively, and incubated for 3 hours. The transcript level analysis of *cre1-96* was performed by qPCR using *sar1* and *act* genes for data normalization. Levels refer to the wild-type strain incubated without carbon source. The values are means from measurements in triplicates and three biological experiments (cultivations). Error bars indicate standard deviations. **(b-d)** *In vivo* and *in vitro* footprinting analysis of URRs of CREI-target genes in Rut-C30, which was pre-grown on glycerol and then incubated on D-glucose (G) or sophorose (SO) for 3 hours followed by DMS-induced *in vivo* methylation. An URR bearing functional CREI sites (underlined in red) of the *cbh1* **(b)**, *cbh2* **(c)**, and *xyr1* **(d)** genes each was investigated, and methylated, naked DNA (ND) was used as the reference. Numbers indicate the position of the base upstream from ATG. Analysis of data and visualization was performed using ivFAST [27]. Colour codes are the same as in Figure 2.

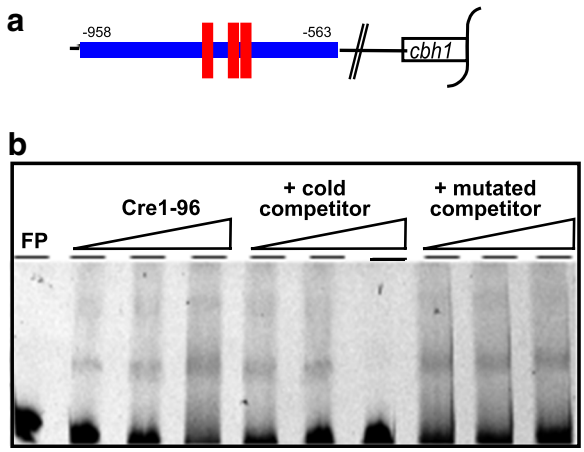
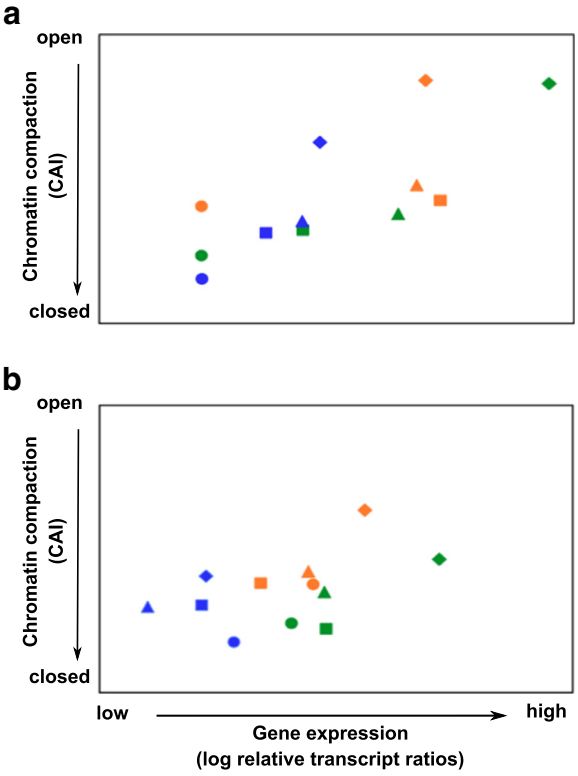


Figure 4 Analysis of the DNA-binding ability of the CREI-96 protein. (a) The EMSA was performed with a fluorescently labelled *cbh1* URR fragment (4 ng) bearing three functional CREI-sites (red bars). **(b)** Applied were increasing amounts of the heterologously expressed and thrombin-cleaved CREI-96 protein (500, 1000, and 2000 nM), and 2000 nM of CREI-96 together with increasing amounts (50-fold, 100-fold, and 200-fold) of a cold competitor (unlabeled probe), and 2000 nM CREI-96 together with increasing amounts (50-fold, 100-fold, and 200-fold) of a mutated cold competitor. FP, free probe.



c

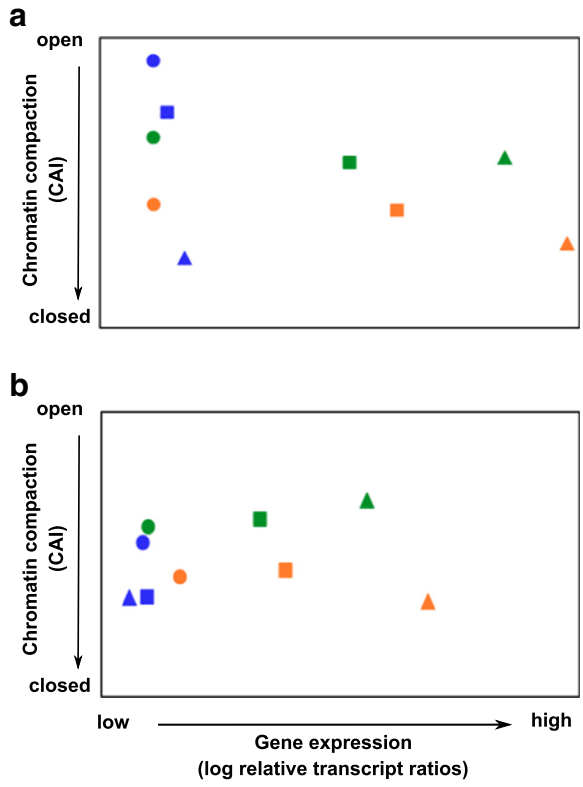
Gene	Spearman correlation	
	QM6a-CREI$_{96}$	QM6aΔ*cre1*
cbh1	0.94 (*P*<0.001)	0.47 (*P*=0.1161)
cbh2	0.35 (*P*=0.2714)	0.49 (*P*=0.0636)
xyr1	0.91 (*P*<0.001)	-0.38 (*P*=0.2705)

Figure 5 Transcript and CHART analysis of *Trichoderma reesei* CREI-mutant strains during growth on D-glucose. QM6a-CREI$_{96}$ **(a)** and the Δ*cre1*-strain **(b)** were grown on D-glucose and samples were taken after 36 (dots), 39 (boxes), 42 (triangles), 45 (diamonds) hours. The *cbh1* (green), *cbh2* (orange), and *xyr1* (blue) genes were investigated. The gene expression analysis was performed by cDNA synthesis followed by qPCR, and transcript levels (log) are depicted on the x-axis. CHART-PCR was performed by Dnase I digestion followed by qPCR, and CAIs are depicted on the y-axis. In both cases *sar1* and *act* genes were used for data normalization and QM6a-CREI$_{96}$ grown for 36 hours was the reference condition. All values are means from measurements in triplicates and three biological experiments (cultivations). Standard deviations were below 5%. Diagrams are identically scaled. **(c)** Spearman's rank correlation coefficients were calculated (close to −1 indicates negative correlation; close to 0 indicates no linear correlation; close to 1 indicates positive correlation). The *P*-values for each correlation coefficient were calculated to determine significance of data (how different from zero). *P* >0.05 was considered as indicative of no linear correlation.

manner. Therefore, we searched the *T. reesei* genome for chromatin-remodelling proteins. We selected seven proteins, of which the annotation pointed to an involvement in chromatin rearrangement, for further investigation (Table 1). To determine a potential regulatory influence by CREI-96 we investigated their transcript levels in QM6a-CREI$_{96}$ and the Δ*cre1*-strain. Amongst the seven genes we observed one with different transcript levels, namely the gene encoding a helicase-like transcription factor (Protein ID 44747). Here we detected strong up-regulation in QM6a-CREI$_{96}$ compared to the Δ*cre1*-strain, regardless of whether the samples from the D-glucose growth experiment (Figure 7a) or the sophorose replacement (Figure 7b) were investigated. We term the aforementioned gene in this study *htf1* (helicase-like transcription factor 1). It should be noted that in previous reports this gene was called *snf2*, although it is not homologous to SNF2 of *Saccharomyces cerevisiae* [30]. However, the increased expression of *htf1* in QM6a-CREI$_{96}$ might be one reason for the observed change in chromatin.

Discussion

For a long time its lack of the full version of CREI has been considered to be a positive genetic property of Rut-C30 because it leads to a partial carbon catabolite de-repression. However, Rut-C30 still possesses a short version of CREI, CREI-96. During this study it became clear that this protein still has a function and influences the expression of target genes, notably, in a positive manner. We found three ways in which CREI-96 mechanistically exerts its role as a regulatory protein. Firstly, it acts directly on promoters of target genes. The short CREI-96 (lacking one zinc finger) can still bind to target DNA sequences *in vitro*, which leads *in vivo* to a clear protection pattern from DNA methylation at CREI-



c

Gene	Spearman correlation	
	QM6a-CREI$_{96}$	QM6aΔ*cre1*
cbh1	-0.43 (*P*=0.5673)	0.31 (*P*=0.4473)
cbh2	-0.41 (*P*=0.2420)	-0.49 (*P*=0.0691)
xyr1	-0.84 (*P*=0.0375)	0.10 (*P*=0.7278)

Figure 6 Transcript and CHART analysis of *Trichoderma reesei* CREI-mutant strains during sophorose induction. QM6a-CREI$_{96}$ **(a)** and the Δ*cre1*-strain **(b)** were pre-grown on glycerol and thereafter incubated on sophorose for 30 (dots), 90 (boxes), and 180 (triangles) minutes. The *cbh1* (green), *cbh2* (orange), and *xyr1* (blue) genes were investigated. The gene expression analysis was performed by cDNA synthesis followed by qPCR, and transcript levels are depicted on the x-axis. CHART-PCR was performed by Dnase I digestion followed by qPCR, and CAIs are depicted on the y-axis. In both cases *sar1* and *act* genes were used for data normalization and QM6a-CREI$_{96}$ incubated for 30 minutes was the reference condition. All values are means from measurements in triplicates and three biological experiments (cultivations). Standard deviations were below 5%. Diagrams are identically scaled. **(c)** Spearman's rank correlation coefficients were calculated (close to −1 indicates negative correlation; close to 0 indicates no linear correlation; close to 1 indicates positive correlation). The *P*-values for each correlation coefficient were calculated to determine significance of data (how different from zero). $P > 0.05$ was considered as indicative of no linear correlation.

binding sites (compare Figures 2 and 3). Obviously, contacting of CREI-binding sites in target genes by CREI-96 leads to a more open chromatin in the core promoter regions under repressing conditions (D-glucose). As expected, this effect was not observed in such a pronounced manner under inducing conditions (sophorose), as the core promoter there already has an open chromatin. If CREI-96 develops this de-regulating function by simply binding the DNA (possibly in high amounts) and thereby preventing nucleosome assembly, or if it is involved in a more complicated chromatin-DNA interaction mechanism, cannot yet be answered and this calls for further investigation.

Secondly, CREI-96 acts on the chromatin structure in an indirect way. The transcript levels of the *htf1* gene encoding a chromatin-remodelling protein are upregulated in the presence of CREI-96. Notably, in the wild-type strain QM6a bearing the native CREI we found *htf1* downregulated, specifically on D-glucose (data not shown). This is in good accordance with previous studies, in which this gene was reported to be repressed by CREI in the *T. reesei* strain QM9414 [26] and to be lowly expressed in QM6a on D-glucose [31]. It can be speculated that CREI-96 in this case reverses the antagonistic function of CREI on chromatin-remodelling proteins and even supports the opening of chromatin in an indirect way.

Thirdly, the loss of the auto-regulatory function of CREI is very likely. It should be considered that surprisingly high transcript levels of *cre1-96* were detected in Rut-C30 (Figure 3a). In *A. nidulans* it was observed that a low steady-state level of *creA* mRNA can be increased within minutes by adding a repressing carbon source. While a prolonged incubation with the repressing carbon sources then led to auto-repression, the incubation on a de-repressing carbon source maintained the high level [18]. In Rut-C30 the *cre1-96* transcript levels on D-glucose are as high as on sophorose, indicating a loss of the mentioned native auto-repression. This might also be the reason for the generally higher abundance of transcript in Rut-C30 compared to the wild-type strain QM6a which we observed during this study. An alternative or additional explanation for the generally higher abundance of *cre1-96* in Rut-C30 is mRNA stability. This may arise from less stability of the mRNA coding for the full-length protein, or a general increase in mRNA stability speculated to be caused by two mutations (protein ID 110423 and 66895) identified in the direct ancestor of Rut-C30 (NG14) [11]. However, it can be assumed that in Rut-C30 this results in high amounts of CREI-96 present in the cell, which is still able to bind DNA, thereby possibly changing the chromatin status in CREI-target genes.

Even if in previous studies truncated forms of CREI have been investigated, interestingly, none of them reported a positive effect of the shorter CREI version compared to a deletion of CREI. This is most likely due to a different experimental design applied. Nakari-Setälä *et al.* reported similar cellulase activities (volumetrically given) of strains bearing a deletion or a truncation of CREI when grown on lactose [32]. However, the investigated strains exhibited different growth behaviors (biomass formation and CO_2-

Table 1 Investigated candidate genes encoding ATP-dependent chromatin remodelling factors

Protein ID	Annotation[a]	Provisory name
44747	Helicase-like transcription factor HLTF/DNA helicase RAD5, DEAD-box superfamily	*htf1 (snf2)*
57935	Chromatin remodelling complex SWI/SNF, component SWI2 and related ATPases (DNA/RNA helicase superfamily)/ATPase	*snf2-like*
21557	Chromatin remodelling factor subunit and related transcription factors	*rsc8*
57608	Chromatin remodelling complex WSTF-ISWI, small subunit	*isw1*
109526	Chromatin remodelling complex WSTF-ISWI, small subunit	*isw2*
50539	SNF2 family DNA-dependent ATPase	*ino80*
58928	Chromodomain-helicase DNA-binding protein	*cdh1*

[a]according to TRIRE Joint Genome Institute - JGI - *Trichoderma reesei* v2.0 database.

levels) [32]. In order to exclude any growth effects we studied a resting cell system under inducing conditions (sophorose). Such a highly standardized transfer experiment allows determination of the mechanistic influence of an isolated phenomenon (presence of CREI-96 or lack of CREI).

We also investigated growth conditions on D-glucose as this is certainly industrially relevant (enzyme production on high glucose-containing inducers). In this case we also observed differences in transcript levels between the two CREI mutant strains, which is in accordance with the results of a Northern analysis of *cbh1* on glucose by Nakari-Setälä *et al.* [32] and of the *cbh1* transcript level analysis by Ries *et al.* [24].

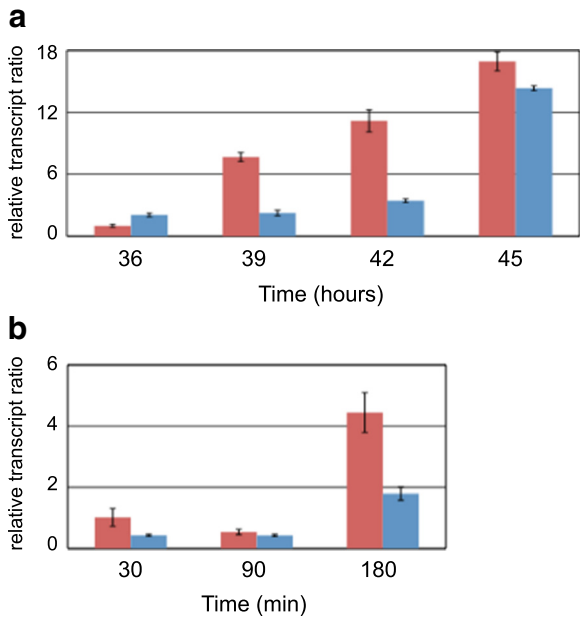


Figure 7 Transcript analysis of *htf1* in *Trichoderma reesei* CREI-mutant strains. QM6a-CREI$_{96}$ (red bars) and the Δ*cre1*-strain (blue bars) were grown on D-glucose and samples were drawn after 36, 39, 42, and 45 hours **(a)** or pre-grown on glycerol and transferred to sophorose for 30, 90, and 180 minutes **(b)**. The transcript levels were analyzed by qPCR using *sar1* and *act* genes for data normalization and levels refer to QM6a-CREI$_{96}$ grown for 36 hours or incubated for 30 minutes, respectively. The values are means from measurements in triplicates and three biological experiments (cultivations). Error bars indicate standard deviations.

We noticed that the increased transcript levels in strain QM6a-CREI$_{96}$ corresponded with an opening of chromatin on D-glucose, which was not found on sophorose. We propose that this different observation is likely due to the fact that the remodelling of chromatin usually becomes necessary under repressing conditions, while under inducing conditions certain regulatory factors and/or mechanisms have already ensured the open chromatin status. Obviously, the differences between the two CREI mutant strains observed on sophorose are primarily related to induction, chromatin opening, and expression of XYRI. During early-stage induction (30 minutes) the presence of sophorose leads to a rapid and higher chromatin accessibility of *xyr1* in QM6a-CREI$_{96}$, which subsequently results in higher *xyr1* transcript levels (Figure 6). It was previously reported that the level of *xyr1* expression is directly linked to the expression of *cbh1* and *cbh2* [6]. Accordingly, we detected higher *cbh1* and *cbh2* transcript levels in the QM6a-CREI$_{96}$ on sophorose.

Conclusions

From the result obtained during this study we conclude that the truncated version of CREI present in Rut-C30 (CREI-96) should be considered as a discrete transcription factor with different properties than CREI. It acts in a direct manner on target URRs, but also contributes indirectly to a more open chromatin status by regulating a chromatin remodeler. Altogether it should be considered that Rut-C30 not only lacks CREI and the thereby mediated CCR, but also gains the now positively acting regulatory protein CREI-96.

Orthologs of CREI regulate CCR in numerous filamentous fungi including those used in biofuel production. Unfortunately, a simple deletion of *cre1* does not only lead to the desired release from CCR, but also to severe growth impairment. Consequently, molecular strain design can alternatively be based on the described CREI-96 truncation. This strategy does on one hand avoid growth

deficiencies and the accompanying loss of productivity, and on the other hand additionally leads to a chromatin remodelling effect that results in increased expression of PCW-degrading enzymes.

Materials and Methods

Fungal strains

The following *T. reesei* strains were used throughout this study: the wild-type strain QM6a (ATCC 13631), Rut-C30, which was described as a high yielding cellulase mutant of QM6a (ATCC 56765) [10], a *cre1* deletion in QM6a (Δ*cre1*) [32], a 83 kb deletion corresponding to the large deletion in scaffold 15 of Rut-C30 in QM6aΔ*tmus53* (Δscaff15) [13,33], as well as a QM6aΔ*tmus53* strain bearing the truncated *cre1* of Rut-C30 (QM6a-CREI$_{96}$) constructed during this study. All strains were maintained on malt extract agar or potato-dextrose-agar.

Growth conditions

For carbon source replacement experiments mycelia were pre-cultured in 1 L Erlenmeyer flasks on a rotary shaker (180 rpm) at 30°C for 24 hours in 250 mL of Mandels-Andreotti (MA) medium [34] supplemented with 1% (w/v) glycerol as sole carbon source. A total of 10^9 conidia per litre (final concentration) were used as inoculum. Pre-grown mycelia were washed and equal amounts were resuspended in 20 ml MA media containing 1% (w/v) D-glucose or 2 mM sophorose (Serva Electrophoresis, Heidelberg, Germany) as sole carbon source, or no carbon source respectively, and incubated for 30 minutes to 3 hours. For direct cultivation experiments the conidia were incubated in 250 mL Erlenmeyer flasks on a rotary shaker (180 rpm) at 30°C for 45 hours in 50 mL of MA medium supplemented with 1% (w/v) D-glucose as sole carbon source. Samples were derived from three biological replicates and were pooled before RNA extraction and chromatin digestion.

Replacement of CREI in *T. reesei* QM6a

Transformation of *T. reesei* QM6aΔt*mus53* [33] was performed using two overlapping 3 kb-DNA fragments. The first fragment consisted of the truncated *cre1* of Rut-C30 amplified with primers RG186 and RG187 using genomic DNA as template and 3′-half of the expression cassette bearing the *Escherichia coli hph* marker gene amplified with primers RG188 and hph3′_fw using the plasmid pRLMex$_{30}$ [35] as template. The second fragment consisted of the 5′-half of the expression cassette bearing the *E. coli hph* marker gene amplified with primers hph5′_rev and RG189 using pRLMex$_{30}$ as template and the *cre1* 3′-flank from Rut-C30 amplified with primers RG190 and RG191 using genomic DNA as template. Protoplast transformation of QM6a was performed as described in United States patent number 8,323,931 using 2.5 μg of each DNA fragment in a co-transformation. Genomic integration of the full construct (*cre1-96*::*hph*) into the *cre1* locus was confirmed by southern blot analysis and DNA sequencing (Microsynth, Balgach, Switzerland).

In vivo footprinting

In vivo methylation using DMS followed by ligation-mediated PCR was performed as described previously [27]. FAM (fluorescein amidite)-labelled fragments were analyzed by capillary gel electrophoresis (Microsynth) and results were analyzed using ivFAST [27].

Analysis of transcript levels

Fungal mycelia were homogenized in 1 mL of peqGOLD-TriFast DNA/RNA/protein purification system reagent (PEQLAB Biotechnologie, Erlangen, Germany) using a FastPrep(R)-24 cell disrupter (MP Biomedicals, Santa Ana, California, United States). RNA was isolated according to the manufacturer's instructions, and the concentration was measured using the NanoDrop 1000 (Thermo Scientific, Waltham, Massachusetts, United States). Synthesis of cDNA from mRNA was carried out using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, Massachusetts, United States) according to the manufacturer's instructions. Quantitative PCRs were performed in a Rotor-Gene Q system (Qiagen, Hilden, Germany). All reactions were performed in triplicate. The amplification mixture (final volume 15 μL) contained 7.5 μL 2 × iQ SYBR Green Mix (Bio-Rad, Hercules, USA), 100 nM forward and reverse primer, and 2.5 μL cDNA (diluted 1:20). Primer sequences are provided in Table 2. Cycling conditions and control reactions were performed as described previously [36]. Data normalization using *sar1* and *act* as reference genes and calculations were performed as published previously [36].

Plasmid construction

A 307 bp fragment was amplified from Rut-C30 genomic DNA using primers RG182 and RG178 and was inserted into the expression vector pGEX-4 T-2 (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom) via *BamH*I/*Xho*I digestion yielding the plasmid pGEX-cre1-RG182 for heterologous expression of GST (glutathione S-transferase)-fused CREI-96.

Protein expression and purification

E. coli BL21(DE3)pLysS (Promega, Madison, Wisconsin, United States) carrying pGEX-cre1-RG182 was cultivated in a 1 L Erlenmeyer flask on a rotary shaker (200 rpm) at 37°C in 300 mL LB medium supplemented with ampicillin (50 μg/mL) until an OD_{600} of 0.5 was reached. Protein expression was induced by adding IPTG to a final concentration of 0.5 mM followed by an incubation at 37°C for 3 hours. The cells were harvested by centrifugation and

Table 2 Oligonucleotides used in this study

Name	Sequence (5′ - 3′)	Usage
RG53	GAATTCAGATC	iv-FP, oligo-short
RG54	GCGGTGACCCGGGAGATCTGAATTC	iv-FP, oligo-long
RG89	[6-FAM]GTAGAGGCATGTTGTGAATCTGTGTCGGG	iv-FP, cbh1oligo3fw, EMSA
RG90	[6-FAM]GGTTGTATGCAAAACGCTCCGAGTCAGAC	iv-FP, cbh1oligo3rev, EMSA
RG215	CCAACGGCTT*GTGGGG*TTGCAGAAGCAACGGCAAAG*CCCCAC*TTCCCC*AC*GTTTGTTTCT[a]	EMSA
RG216	AGAAACAAAC*GTGGGGAAGTGGGG*CTTTGCCGTTGCTTCTGCAA*CCCCAC*AAGCCGTTGG	
RG221	CCAACGGCTT*GT**T**GGG*TTGCAGAAGCAACGGCAAAG*CCC**A**AC*TTCCC**A**AC*GTTTGTTTCT[b]	
RG222	AGAAACAAAC*GT**T**GGGAAGT**T**GGG*CTTTGCCGTTGCTTCTGCAA*CCC**A**AC*AAGCCGTTGG	
RG178	TATCTCGAGTTTAGAAAAAAAAGCAGGT	pGEX-cre1-RG182 construction
RG182	ATTGGATCCATGCAACGAGCACAGTCTGCCGT	
RG186	TTGAGTGCAGACGTGTGTGTAATCTT	Construction of QM6a-CREI$_{96}$
RG187	CCCTCCTTTGTTAGAAAAAAAAGCAGGTAATGG	
RG188	TTTTTTCTAACAAAGGAGGGAGACGAGGTTG	
RG189	CCTACATTGGATAACGGTGAGACTAGCGGCC	
RG190	TCACCGTTATCCAATGTAGGTAAGTAGTAAGGG	
RG191	GAATCAGTATTTTCTCATCTCCTTG	
hph3′_fw	GACCTGCCTGAAACCGAACTG	
hph5′_rev	GAAGAAGATGTTGGCGACCTCG	
actfw	TGAGAGCGGTGGTATCCACG	qPCR
actrev	GGTACCACCAGACATGACAATGTTG	
sar1fw	TGGATCGTCAACTGGTTCTACGA	
sar1rev	GCATGTGTAGCAACGTGGTCTTT	
cbh1f	GATGATGACTACGCCAACATGCTG	
cbh1r	ACGGCACCGGGTGTGG	
cbh2f	CTATGCCGGACAGTTTGTGGTG	
cbh2r	GTCAGGCTCAATAACCAGGAGG	
xyr1f	CCCATTCGGCGGAGGATCAG	
xyr1r	CGAATTCTATACAATGGGCACATGGG	
44747f	GCTCGAGCTGCAAGACAAGA	
44747r	GCGGAGATCCATGAGCTTCT	
epiactinTr_f	CTTCCCTCCTTTCCTCCCCCTCCAC	act CHART, region −226 to +24
epiactinTr_r	GCGACAGGTGCACGTACCCTCCATT	
episar1Tr_f	GTCAGGAAATGCCGCACAAGCAAGA	sar1 CHART, region −490 to −224
episar1Tr_r	TGTGTTTTACCGCCTTGGCCTTTGG	
epicbh1_2Tr_f	GGATCGAACACACTGCTGCCTTTAC	cbh1 CHART, region −301 to −27
epicbh1_2Tr_r	GGTTTCTGTGCCTCAAAAGATGGTG	
epicbh2_2Tr_f	TGCAGCGCAACACTACACGCAACAT	cbh2 CHART, region −355 to −62
epicbh2_2Tr_r	TGCGCCTCATACAGGGTCACAGTCC	
epixyr1_2Tr_f	CCGACAGCAGCAGTAGTCAGGTTTT	xyr1 CHART, region −216 to +35
epixyr1_2Tr_r	TAGGCAGAATAGCGACGGAGAGGAT	

[a]Italic letters indicate a CREI-binding site (5′-SYGGRG-3′).
[b]Bold letters indicate the introduced mutation in the CREI-binding site (5′-SYTGRG-3′).

stored at −20°C overnight. GST-fusion protein (theoretical molecular weight 37 kDa) was purified from *E. coli* cell lysates using Glutathione-Superflow Resin (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

A 400-bp PCR product comprising the part of the 5′-URR region of *cbh1*, which contains 3 CREI-binding sites, was obtained with FAM-labelled primers RG89 and RG90 and used as probe (Table 2). The protein-DNA binding assay and non-denaturing polyacrylamide gel electrophoresis were performed essentially as previously described [37]. Binding was achieved by incubating increasing amounts of heterologously expressed, thrombin-cleaved CREI-96 (500 nM, 1000 nM, and 2000 nM) with 4 ng of the labelled, double-stranded DNA fragment in GST elution buffer (10 minutes at 22°C). Fluorescence and image analysis of the gels was carried out using a Typhoon 8600 variable mode imager (Amersham Bioscience, part of GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Competition experiments were performed using 2000 nM of protein together with increasing amounts (50-fold, 100-fold, and 200-fold) of either a cold competitor (an unlabeled double-stranded DNA probe obtained by annealing primers RG215 and RG216) or a mutated cold competitor (an unlabeled double-stranded DNA probe obtained by annealing primers RG221 and RG222). This probe bears in all three CREI-binding sites a mutation from 5′-SYGGRG-3′ to 5′-SYTGRG-3′, which was previously shown to prevent the binding of CREI *in vivo* and *in vitro* [21].

Determination of cellulase activity

Cellulase activity in the culture supernatants was determined using AZCL HE-Cellulose (Megazyme International, Bray, Ireland) in 25 mM sodium acetate buffer pH 4.5 according to the manufacturer's instructions. To measure biomass (dry weight), the cultures were harvested by filtration, washed with an equal volume of 0.8% NaCl solution, dried at 80°C for 24 hours, and weighed. Samples from two biological replicates and two technical replicates were measured.

Chromatin accessibility real-time PCR (CHART-PCR)

DNase I digestion of chromatin and DNA extraction were carried out as described by Gonzalez and Scazzocchio [38] with minor modifications. Mycelia were harvested by filtration, pressed dry with filter paper, frozen in liquid nitrogen, and ground to a fine powder. Portions (100 mg) of the powder were suspended in 1 mL aliquots of nuclease digestion buffer (250 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.05 mM $CaCl_2$, 3 mM $MgCl_2$, 0.5 mM DTT, and 15 mM Tris-HCl at pH 7.5), and 100-μL samples of the digestion mixture were incubated with 10 U of RQ1 RNase-free DNase I (Promega, Madison, Wisconsin, United States) for 2.5 minutes at 37°C. The reaction was stopped by adding 100 μL of 40 mM EDTA and 2% SDS, followed by two rounds of phenol-chloroform extraction and one round of chloroform extraction. Samples were then treated with 10 μg/mL of RNase A for 15 minutes at 37°C and precipitated with ethanol. DNA pellets were suspended in 100 μL of 5 mM Tris-HCl at pH 7.5. A control without DNase I was included to monitor endonuclease activity. qPCR analysis of the DNase I-treated samples was performed to measure the relative abundance of target regions. PCRs were performed in a Rotor-Gene Q system (Qiagen, Hilden, Germany). All reactions were performed in triplicate. The amplification mixture (final volume 20 μL) contained 10 μL 2 × iQ SYBR Green Mix (Bio-Rad, Hercules, USA), 200 nM forward and reverse primers and 10 ng of DNA. Primer sequences are provided in Table 2. Cycling conditions were as follows: 3 minutes initial denaturation at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The amount of intact input DNA of each sample was calculated by comparing the threshold values of the PCR amplification plots with a standard curve generated for each primer set using serial dilutions of genomic, undigested DNA. The chromatin accessibility index (CAI) was defined as:

$$\mathrm{CAI} = 1/(\mathrm{Ds}/((\mathrm{Dc1} + \mathrm{Dc2})/2)) \quad (1)$$

where Ds is the amount of intact DNA detected for each target region and Dc1 and Dc2 are the amounts of intact DNA detect for the promoter regions of *sar1* and *act* respectively, used as reference genes for normalization.

To access the relationship between the CAI and the transcript level, a two-tailed Spearman's rank correlation coefficient was determined for each gene analyzed. The *P*-value for each correlation coefficient was calculated to determine the significance of the data.

Selection of investigated genes encoding chromatin remodelling factors

Genes were selected by direct searching for SNF2, ISW1, ISW2, INO80, CDH1, RSC8 (the most prominent chromatin remodelling-related proteins characterized in yeast) in the *T. reesei* genome database [39]. Additional candidate genes were obtained by using BLASTp (basic local alignment search tool) search in the NCBI database employing respective *S. cerevisiae* sequences as baits to identify similar sequences in filamentous fungi. Subsequently, these sequences were used in a BLAST search in the *T. reesei* database.

Abbreviations

BLAST: Basic local alignment search tool; CAI: Chromatin accessibility index; CHART-PCR: Chromatin accessibility real-time PCR; CREI: Carbon catabolite repressor 1; DMS: Dimethyl sulphate; EMSA: Electrophoretic mobility shift assay; GST: Glutathione S-transferase; ivFAST: *in vivo* footprinting analysis

software tool; MA: Mandels-Andreotti; PCW: Plant cell wall; RT-qPCR: Reverse transcription quantitative PCR; URR: Upstream regulatory region; XYRI: Xylanase regulator 1.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RG constructed the strain bearing the truncated version of CREI. TMMS carried out transcript analyses, CHART-PCR, and EMSA. AR carried out the *in vivo* footprinting analyses. MJPF participated in conception of the study and revised the manuscript critically. RLM participated in conception of the study. ARMA participated in conception of the study, supervised the experiments, and prepared the manuscript. All authors read and approved the final manuscript.

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The impact of chromatin remodelling on cellulase expression in *Trichoderma reesei*

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Abstract

Background: *Trichoderma reesei* is used for industry-scale production of plant cell wall-degrading enzymes, in particular cellulases, but also xylanases. The expression of the encoding genes was so far primarily investigated on the level of transcriptional regulation by regulatory proteins. Otherwise, the impact of chromatin remodelling on gene expression received hardly any attention. In this study we aimed to learn if the chromatin status changes in context to the applied conditions (repressing/inducing), and if the presence or absence of the essential transactivator, the Xylanase regulator 1 (Xyr1), influences the chromatin packaging.

Results: Comparing the results of chromatin accessibility real-time PCR analyses and gene expression studies of the two prominent cellulase-encoding genes, *cbh1* and *cbh2,* we found that the chromatin opens during sophorose-mediated induction compared to D-glucose-conferred repression. In the strain bearing a *xyr1* deletion the sophorose mediated induction of gene expression is lost and the chromatin opening is strongly reduced. In all conditions the chromatin got denser when Xyr1 is absent. In the case of the xylanase-encoding genes, *xyn1* and *xyn2,* the result was similar concerning the condition-specific response of the chromatin compaction. However, the difference in chromatin status provoked by the absence of Xyr1 is less pronounced. A more detailed investigation of the DNA accessibility in the *cbh1* promoter showed that the deletion of *xyr1* changed the *in vivo* footprinting pattern. In particular, we detected increased hypersensitivity on Xyr1-sites and stronger protection of Cre1-sites. Looking for the players directly causing the observed chromatin remodelling, a whole transcriptome shotgun sequencing revealed that 15 genes encoding putative chromatin remodelers are differentially expressed in response to the applied condition and two amongst them are differentially expressed in the absence of Xyr1.

Conclusions: The regulation of xylanase and cellulase expression in *T. reesei* is not only restricted to the action of transcription factors but is clearly related to changes in the chromatin packaging. Both the applied condition and the presence of Xyr1 influence chromatin status.

Background

In nature, *Trichoderma reesei* is as a saprophytic fungus an excellent producer of enzymes involved in plant cell wall degradation (PCWD). In industry, these enzymes are used for a number of applications: xylanases are used for example in food industry as a baking agent and for clarification of juice and wine [1] or in the paper industry for de-inking [2]. Cellulases from *T. reesei* are important in textile industry for example for fibre polishing [3] or in the paper industry for recycling processes [2]. In the production of ethanol from cellulosic raw material *T. reesei* enzymes are applied to break down lignocellulose material to release D-glucose. The obtained D-glucose can be used subsequently in the sugar-to-ethanol fermentation (e.g. [4, 5] and citations therein). Due to the multiple applications of these enzymes many research studies have focused on this organism, its PCWD enzyme expression, and finally, the regulation of the encoding genes. Most of these studies were performed in the wild-type strain QM6a [6] or the mutant strain QM9414, which was selected for increased cellulase production [7]. Genome-wide analyses identified

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34 cellulolytic and xylanolytic enzyme-encoding genes in *T. reesei* (reviewed in [8]), of which the most prominent cellulases are the cellobiohydrolases CBHI and CBHII (EC 3.2.1.91) [9] and the most studied xylanases are the endo-ß-1,4-xylanases XYNI and XYNII (EC 3.2.1.8) [10]. The mentioned research efforts led further to the identification of transcription factors involved in the regulation of the expression of genes coding for PCWD enzymes on the transcriptional level. The most important transactivator is the Xylanase regulator 1 (Xyr1), which is absolutely essential for expression of both, xylanase and cellulase-encoding genes [11]. However, it should be noted that only the cellulase expression strictly follows the induction/repression pattern of the *xyr1* gene [12]. The *xyr1* gene itself is usually expressed at a low level and can be induced by the disaccharide sophorose formed via transglycosylation [12, 13]. Otherwise, the xylanase expression depends on Xyr1, but the transcript levels of these genes do not strictly reflect *xyr1* transcript levels [11, 12]. The most important repressor is the Carbon catabolite repressor 1 (Cre1) [14], which mediates carbon catabolite repression (CCR) in presence of high amounts of easily usable carbon sources, such as D-glucose or D-xylose. Cre1 exerts its repressing function on both, the genes coding for the PCWD enzymes and the gene coding for their activator, *xyr1* (e.g. [13, 15]). The different response of *T. reesei*'s transcriptome and secretome to cellulose, sophorose, and D-glucose was just recently investigated in a comparative high-throughput genomic and proteomic study [16]. While a lot is known about the transcriptional regulation of *T. reesei*'s PCWD enzyme-encoding genes by regulatory proteins (reviewed in [17]), so far hardly anything was investigated concerning the impact of the chromatin status on their gene expression. Only for Cre1 it was already earlier suggested that it might influence chromatin remodelling [18]. More recently, it was reported that it is involved in nucleosome positioning [19], and that a truncated version of Cre1, which is present in CCR-released, cellulase hyper-producing strains, supports the opening of chromatin in Cre1-target genes [20]. However, taking into account that chromatin status generally is believed to be a crucial factor in gene expression, this topic did not receive much attention in *T. reesei* yet. Therefore, in this study, we aimed to learn if the opponent of Cre1, the transactivator Xyr1, is also involved in chromatin remodelling, and if this happens in a condition (inducing/repressing carbon source)-dependent way. We used chromatin accessibility real-time PCR (CHART-PCR) for determining the chromatin status of the genes encoding the mentioned, four major PCWD enzymes and compared this with their gene expression. The results prompted us to have a more detailed investigation of the *cbh1* promoter by *in vivo* footprinting analyses. Finally, we used whole transcriptome shotgun sequencing (WTSS) to identify genes putatively involved in chromatin remodelling that are differentially expressed with regards to the applied condition and/or the absence or presence of Xyr1.

Results

Decreased cellulase gene expression in the absence of Xyr1 goes along with denser chromatin

It is well known that Xyr1 is an essential activator of cellulase gene expression [11]. However, so far it has not been investigated if the deletion of Xyr1 additionally influences the chromatin status in the fungus. In order to study this, the wild-type strain and the *xyr1* deletion strain were pre-grown and transferred to sophorose (inducing condition), D-glucose (repressing condition) or no carbon source-containing medium (reference condition) and were incubated for 3 h. By applying CHART-PCR analysis we investigated the chromatin packaging of the core promoter region (bearing the TATA-box) and one upstream regulatory region (URR) bearing Xyr1-binding sites (5′-GGC(T/A)$_3$-3′; [21]) and/or Cre1-binding sites (5′-SYGGRG-3′; [14]) of the *cbh1* and *cbh2* genes each. For overviews on the investigated regions see Fig. 1a, b. Supplementary, we investigated the transcript levels of these genes by reverse transcription, quantitative PCR (qPCR) to see if the expression is related to chromatin accessibility. The expression of *cbh1* and *cbh2* is repressed on D-glucose in both strains and induced by sophorose in the wild-type strain (Fig. 2a, b). The induction is lost in the *xyr1* deletion strain aside from a small increase in gene expression on sophorose compared to D-glucose. Altogether, we observed in both strains a condition-dependent change (i.e. sophorose-mediated opening) of chromatin that went along with a change (i.e. sophorose-mediated increase) in gene expression. However, comparing the strains under the same condition, the chromatin was always more closed in the *xyr1* deletion strain compared to the wild-type strain (Fig. 2a, b) indicating a contribution of Xyr1 to a general (i.e. condition-independent) opening of chromatin in upstream regions of the cellulase-encoding genes.

Xylanase gene repression in the absence of Xyr1 is not strictly related to chromatin compaction

In an analogous analysis we investigated the chromatin status of the core promoter and an URR of the *xyn1* and *xyn2* genes each and compared this to the expression of the respective genes. For overviews on the regions investigated by CHART-PCR see Fig. 1c, d. In the wild-type strain the repression on D-glucose, the basal expression on D-xylose, and the induction on sophorose coincided with the increasing opening of chromatin (Fig. 3a, b). Otherwise, in the *xyr1* deletion strain the gene

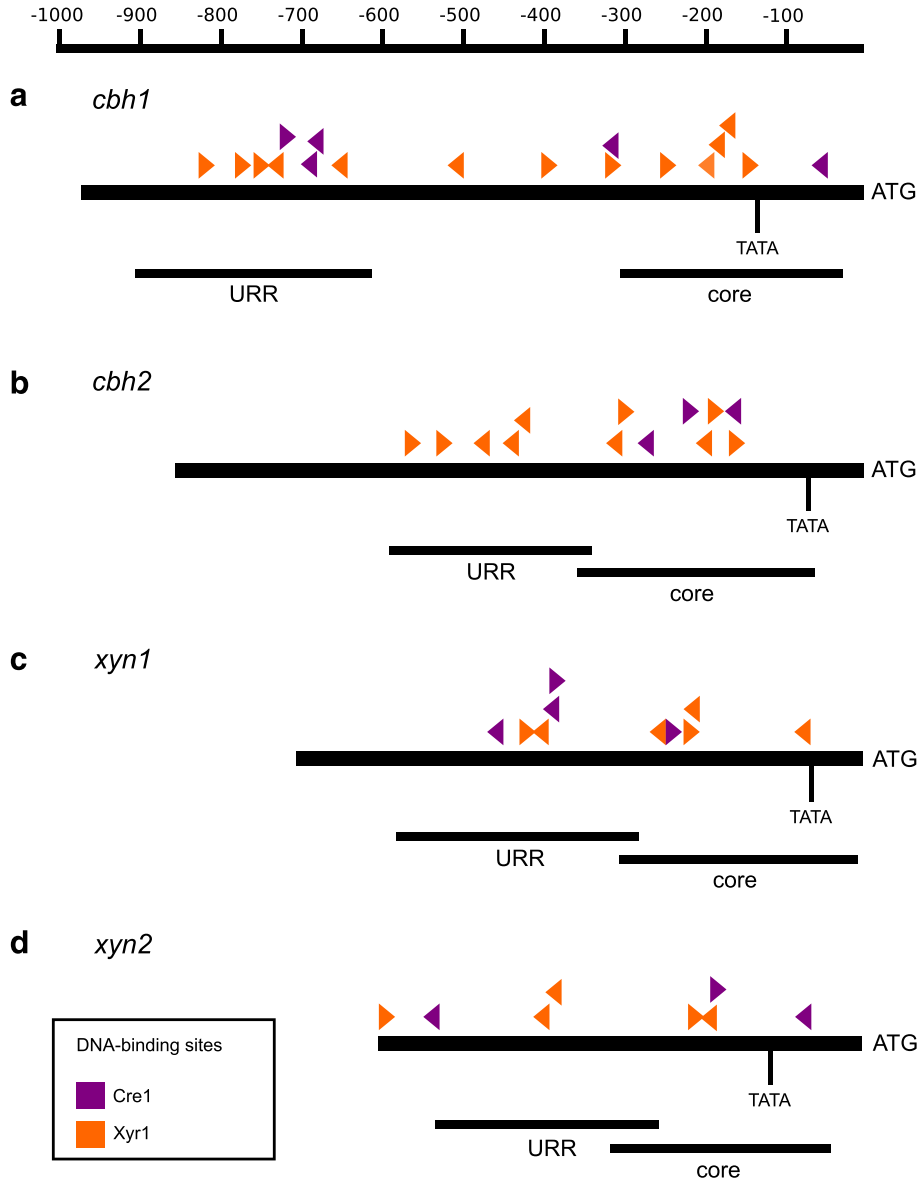

Fig. 1 Overview on the upstream sequence of the investigated genes encoding PCWD enzymes. The regions investigated by CHART-PCR are indicated by black bars. The core promoter region covering the TATA-box (core) and an URR of the *cbh1* (**a**), *cbh2* (**b**), *xyn1* (**c**), and *xyn2* (**d**) genes each are depicted. DNA-binding sites of Xyr1 and Cre1 are indicated by orange and purple triangles, respectively. The orientation of the triangle represents the orientation of the binding motif. The scale at the top indicates distance from ATG in bp

expression was at a similar low level (repressed) independent from the tested condition, while the chromatin packaging differed between the conditions. Interestingly, the chromatin accessibility on sophorose was even similar between the Δ*xyr1*-strain and the wild-type strain (except the URR of *xyn1*) but the sophorose-mediated induction was completely lost in the Δ*xyr1*-strain (Fig. 3a, b). Summarizing, we detected - similar to the case of the cellulase-encoding genes - an induction-specific opening of chromatin together with increasing gene expression in the wild-type strain. However, different from the cellulases, xylanase expression was repressed in the Δ*xyr1*-strain although the chromatin status differed condition-dependently.

Contribution of Xyr1 to chromatin opening

To understand in detail the contribution of Xyr1 to changes in chromatin packing, the relation to induction of gene expression, and its putative impact on transcription initiation, we used CHART analysis again. We compared samples from the *T. reesei* wild-type and the *xyr1* deletion strain exposed to sophorose (inducing condition) and to

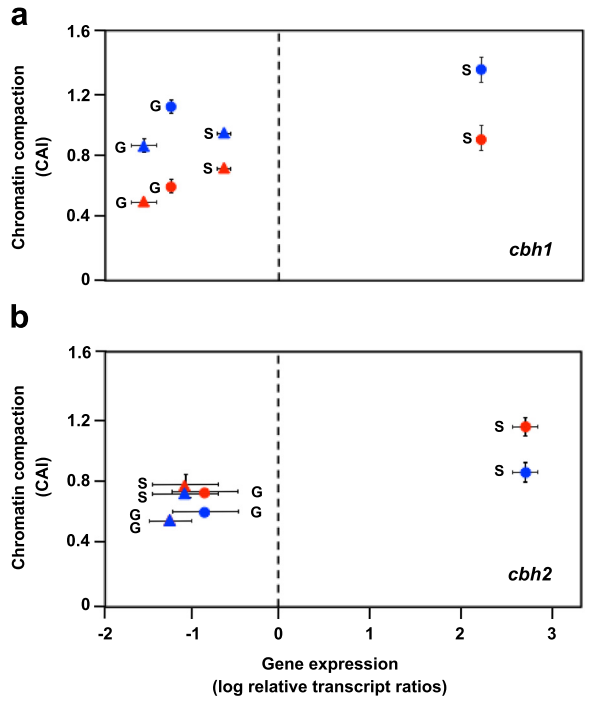

Fig. 2 Transcript and CHART analysis of cellulase-encoding genes in the presence or absence of Xyr1. The *T. reesei* wild-type strain (dots) and the Δ*xyr1*-strain (triangles) were pre-grown on glycerol and thereafter incubated on D-glucose (G) or sophorose (S) for 3 h. The core promoter region (*red*) and an URR (*blue*) of *cbh1* (**a**) and *cbh2* (**b**) genes were investigated. The gene expression analysis was performed by cDNA synthesis followed by qPCR, and transcript levels are depicted on the x-axis. CHART-PCR was performed by DNaseI digestion followed by qPCR, and chromatin accessibility indices (CAIs) are depicted on the y-axis. In both cases *sar1* and *act* genes were used for data normalization and the wild-type strain incubated without carbon source for 3 h was the reference condition. The dashed line indicates transcript level of the reference condition, i.e. levels above are considered induced and levels below are considered repressed. All values are means from measurements in triplicates and three biological experiments (cultivations). The error bars indicate standard deviations. Diagrams are identically scaled

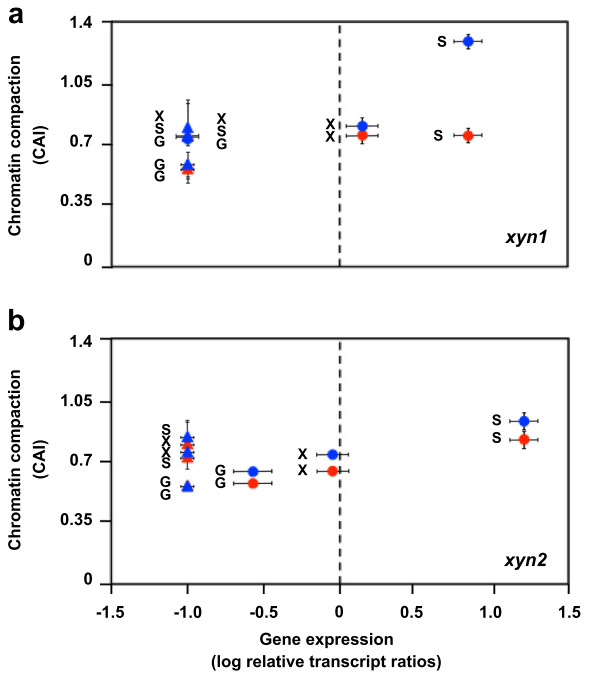

Fig. 3 Transcript and CHART analysis of xylanase-encoding genes in the presence or absence of Xyr1. The *T. reesei* wild-type strain (dots) and the Δ*xyr1*-strain (triangles) were pre-grown on glycerol and thereafter incubated on D-glucose (G), D-xylose (X) or sophorose (S) for 3 h. The core promoter region (*red*) and an URR (*blue*) of *xyn1* (**a**) and *xyn2* (**b**) genes were investigated. The gene expression analysis was performed by cDNA synthesis followed by qPCR, and transcript levels are depicted on the x-axis. CHART-PCR was performed by DNaseI digestion followed by qPCR, and CAIs are depicted on the y-axis. In both cases *sar1* and *act* genes were used for data normalization and the wild-type strain incubated without carbon source for 3 h was the reference condition. The dashed line indicates transcript level of the reference condition, i.e. levels above are considered induced and levels below are considered repressed. All values are means from measurements in triplicates and three biological experiments (cultivations). The error bars indicate standard deviations. Diagrams are identically scaled

non carbon source (non inducing condition). In the wild-type strain chromatin opens specifically on sophorose in case of all tested genes, namely *xyn1*, *xyn2*, *cbh1*, and *cbh2* (Fig. 4). This is lost for all genes in the Δ*xyr1*-strain (Fig. 4). However, the induction specific opening of chromatin is more pronounced in case of the cellulase-encoding genes. Altogether, the comparison of the chromatin accessibility under induced and non-induced conditions in the wild-type and the Δ*xyr1*-strain even suggested that the open status is a consequence of induction. Xyr1 is required for the chromatin loosening, but this action is not essential for the initiation of transcription because transcripts can also be detected at low levels in a *xyr1* deletion strain (compare Figs. 2 and 3).

The absence of Xyr1 changes DNA accessibility in the *cbh1* promoter

Since we observed a pronounced induction-specific opening of chromatin that went along with increase of gene expression in presence of Xyr1 and a closing of chromatin together with gene repression in the absence of Xyr1 in the case of the cellulase-encoding genes, we aimed to have a more detailed investigation on the DNA accessibility of the promoter. Therefore, we performed *in vivo* footprinting analyses of the *cbh1* promoter. Two URRs bearing Xyr1-binding sites and/or Cre1-binding sites and the core promoter bearing Xyr1-binding sites close to the TATA-box were investigated (Fig. 5a). The wild-type strain and the Δ*xyr1*-strain were pre-grown on

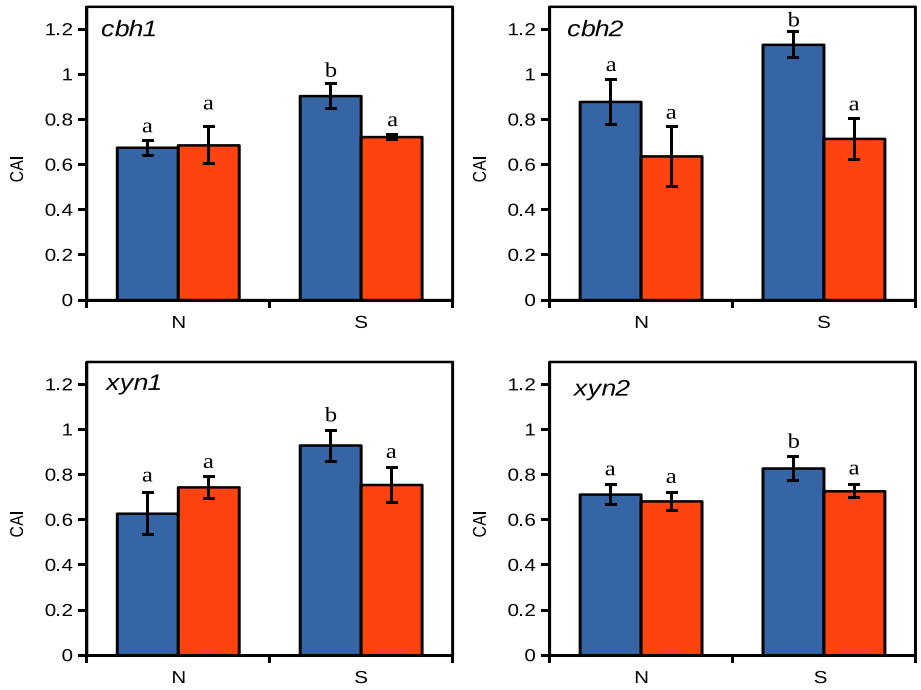

Fig. 4 CHART analysis of cellulase- and xylanase-encoding genes in presence and. The *T. reesei* wild-type strain (*blue bars*) and the Δ*xyr1*-strain (*red bars*) were pregrown on glycerol and thereafter incubated without carbon source (N) or in presence of 2.0 mM sophorose (S) for 3 h. The core promoter regions of *cbh1*, *cbh2*, *xyn1*, and *xyn2* genes were investigated. CHART-PCR was performed by DNaseI digestion followed by qPCR using sar1 and act genes were for data normalization. Chromatin accession indices (CAI) are depicted on the y-axis. All values are means from measurements in triplicate and three biological experiments (cultivations). The error bars depict the standard deviation and different letters denote statistical difference among compared data employing t-test ($P < 0.05$)

glycerol and then incubated on D-glucose or sophorose for 3 h followed by dimethyl sulphate (DMS)-induced *in vivo* methylation. From Fig. 5b–d the footprinting pattern of the *xyr1* deletion strain compared to the wild-type strain for the three investigated regions can be inferred. The first investigated URR bears next to a single Cre1-site and a single Xyr1-site, also two Xyr1-sites arranged as inverted repeat with a spacing of 11 bp, which was reported to be the functional binding motif *in vivo* [22]. Under both, repressing and inducing conditions we could detect strong differences in the footprinting pattern of the two strains (Fig. 5b). In particular on sophorose, we observed an increased hypersensitivity towards DNA methylation on the Xyr1-sites in the Δ*xyr1*-strain compared to the wild-type strain, whereas the Cre1-site was stronger protected (Fig. 5b). The second investigated URR bears a functional Cre1 double site [23]. Here, we detected strong hypermethylation signals in the Δ*xyr1*-strain compared to the wild-type strain on D-glucose, but none on sophorose (Fig. 5c). The third investigated URR bears three Xyr1-binding sites arranged *in tandem*. In this case, we detected just a few differences between the two strains, however, most of them on or close to the Xyr1-sites (Fig. 5d).

Identification of differentially expressed genes potentially involved in chromatin remodelling

To learn more about the mechanisms responsible for the chromatin remodelling in context to both, the applied condition and the presence or absence of Xyr1, we used WTSS. Therefore, a *xyr1* deletion strain and its parental strain QM9414 were again exposed to repressing conditions (growth on D-glucose) and inducing conditions (incubation on sophorose). Please note that the full data set can be obtained from GEO database (GSE66982). Based on the results obtained by the WTSS, we analyzed the gene expression profiles of 136 candidate genes involved in chromatin structure and dynamics according to the eukaryotic orthologous groups (KOG) in the *T. reesei* genome database (http://genome.jgi-psf.org/Trire2/Trire2.home.html). An overview on these 136 genes is provided here. For the differential expression analysis, a two-fold change cut-off, *i.e.* $\log_2$ fold change ≥ 1 or ≤ −1 and an adjusted *p*-value ≤ 0.05, was used as threshold. Concerning the first part of our question, *i.e.* the observed differences in chromatin status dependent on the applied condition, we identified 15 genes differentially expressed on sophorose as compared to D-glucose in the wild-type strain (listed in Table 1).

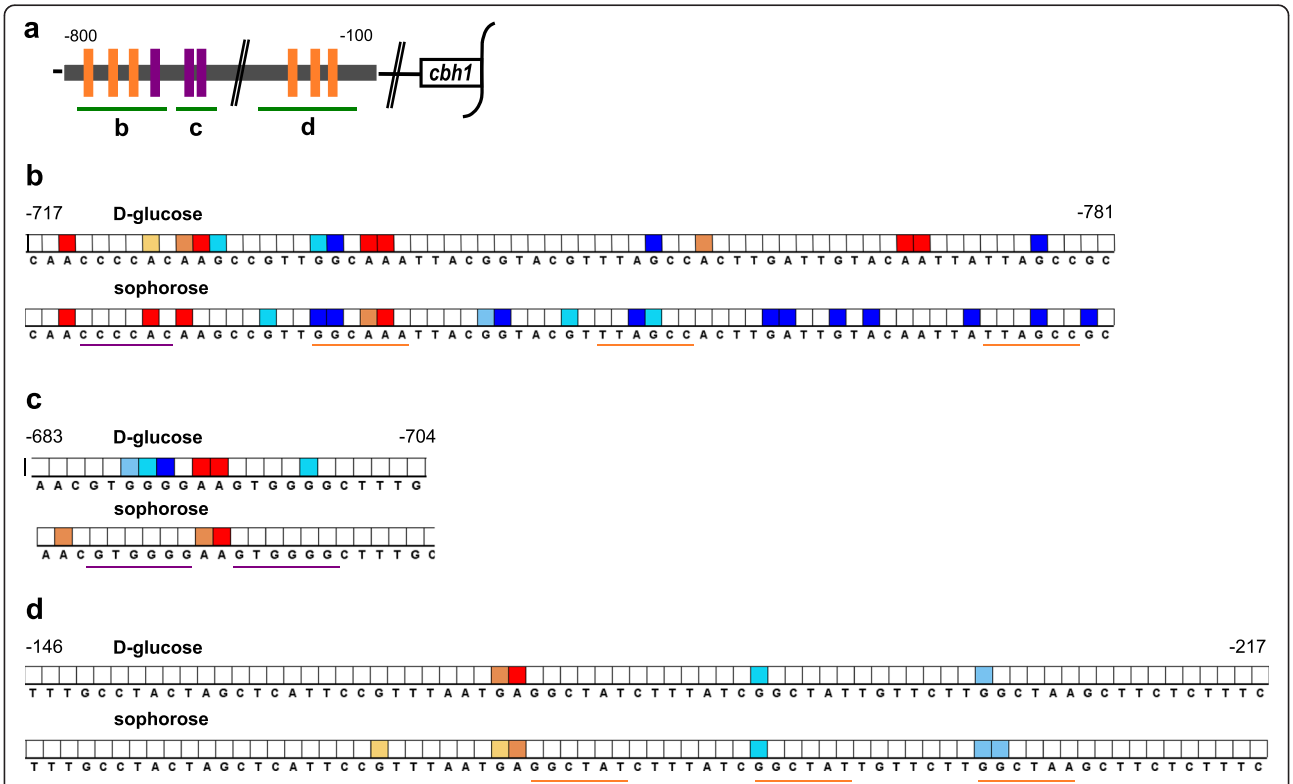

Fig. 5 *In vivo* footprinting analyses of the *cbh1* promoter in the presence or absence of Xyr1. The *T. reesei* wild-type strain QM6a and the Δ*xyr1*-strain were pre-grown on glycerol and then incubated on D-glucose or sophorose for 3 h followed by DMS-induced *in vivo* methylation. **a** Schematic drawing of the *cbh1* promoter and the investigated regions (indicated by *green lines*). Two URRs (**b**, **c**) bearing functional Xyr1-binding sites (*orange*) or Cre1-sites (*purple*) and the core promoter region (**d**) bearing Xyr1-binding sites (*orange*) were investigated on the forward strand. Numbers indicate the position of the base upstream from ATG. Analysis of data and visualization was performed using ivFAST [34]. Only signals that are statistically different are considered; protected bases are highlighted in *red shades* and hypersensitive bases are highlighted in *blue shades*; the three colour intensities each correspond to stronger differences between compared conditions (Δ*xyr1*-strain compared to wild-type strain), i.e. increasing colour intensity means more than 1.1-, 1.3-, and 1.5-fold difference

Concerning, the second part of our question, i.e. the influence of Xyr1 on the expression profiles of these genes we examined which ones were differentially expressed in Δ*xyr1*-strain compared to the wild-type strain under sophorose induction. Out of the 15 genes responding to the applied condition, two genes are additionally differentially expressed in the Δ*xyr1*-strain (transcript ID 53947 and 73708). Notably, the gene with transcript ID 73708, encoding a putative heterochromatin-associated protein, was down-regulated on sophorose compared to D-glucose and up-regulated in the absence of Xyr1.

Discussion

The aim of this study was to learn more about the contribution of the chromatin compaction to the regulation of gene expression of PCWD enzymes in *T. reesei*. Altogether, we found for all investigated genes that their induced expression is accompanied by an opening of chromatin and that Xyr1 is required for the open chromatin status.

However, we observed differences between cellulase- and xylanase-encoding genes concerning the involvement of Xyr1 in chromatin remodelling: the chromatin of the upstream regions of the cellulase-encoding genes was more compact under all tested conditions when Xyr1 was missing. This finding is supported by the *in vivo* footprinting results of the *cbh1* URR, which revealed an increased sensitivity towards methylation on the Xyr1-binding sites in the absence of Xyr1, in particular on sophorose (compare Fig. 4b). On the other hand, the accessibility of the functional Cre1-sites was changed only on D-glucose. They were found to be stronger methylated in the *xyr1* deletion strain than in the parent strain (compare Fig. 4c). We assume that hypersensitivity to DNA methylation can be caused by both, non-occupancy leading to better access for the methylation agent, but also by DNA occupancy and a following increased disposition to be methylated. Considering this, we would suggest that on D-glucose repression the Cre1 DNA-binding affinity to the *cbh1* promoter is higher in the absence of Xyr1. This could explain the less accessible chromatin in the Δ*xyr1*-strain

Table 1 Differentially expressed genes that are potentially involved in chromatin remodelling

Transcript ID	Annotation	SO/G	*p*-value	Δ*xyr1*/WT	*p*-value
2648	Predicted component of NuA3 histone acetyltransferase complex	−1.154	0.000	0.279	0.653
34402	Histone H1	1.300	0.000	−0.457	0.075
36727	SWI-SNF chromatin-remodeling complex protein	1.493	0.000	−0.843	0.000
53947	SWI-SNF chromatin-remodeling complex protein	1.196	0.000	1.332	0.000
56077	SWI-SNF chromatin-remodeling complex protein	3.050	0.000	−0.359	0.158
65533	Histone deacetylase complex, catalytic component HDA1	1.237	0.000	−0.347	0.170
73708	Heterochromatin-associated protein HP1 and related CHROMO domain proteins	−3.012	0.002	1.253	0.000
76872	SWI-SNF chromatin-remodeling complex protein	1.070	0.000	−0.708	0.004
81517	Sirtuin 5 and related class III sirtuins (SIR2 family)	1.600	0.034	−0.160	0.615
108909	Nucleosome-binding factor SPN, POB3 subunit	1.050	0.000	0.041	0.998
110409	Possible homologue of *S. cerevisiae* SAS10	−1.298	0.000	−0.001	1.000
110418	SWI-SNF chromatin-remodeling complex protein	1.180	0.000	−0.566	0.049
110507	Histone acetyltransferase (MYST family)	1.064	0.000	−0.562	0.024
122943	SWI-SNF chromatin-remodeling complex protein	1.876	0.000	−0.127	0.621
123327	SWI-SNF chromatin-remodeling complex protein	1.852	0.002	0.508	0.036

Differential gene expression according to WTSS analysis comparing either sophorose induction (SO) to glucose repression (G) in the wild-type strain or the *xyr1* deletion strain (Δ*xyr1*) to the wild-type strain (WT) under sophorose induction

on D-glucose (compare Fig. 2a). In summary, the presence of Xyr1 supported chromatin opening under all investigated conditions in the case of the cellulase-encoding genes. Perhaps this finding is one explanation for the previously reported, condition-dependent transcript level pattern of the *cbh1* and *cbh2* genes that exactly follow the one of the *xyr1* gene [12]. For example, if under non-inducing conditions less Xyr1 is present, the positive influence of Xyr1 on chromatin opening might be reduced and this would cause a decrease in cellulase-encoding gene expression. However, the earlier observation that transcript levels of *cbh1* and *cbh2* correlate with those of *xyr1* [12], and the result from this study indicating the involvement of Xyr1 in chromatin opening suggest a regulation of the cellulase-encoding genes being dominated by Xyr1.

It is currently thought that transcription factors must induce the reorganization of the local chromatin (reviewed by [24]). One proposed mechanism is the recruitment of nucleosome remodelers by the initiating factor leading to local chromatin conformations [25]. Our current model on the function of the Xyr1 is the following: as shown in previous studies *xyr1* transcription is induced on sophorose [12]. This allows the assumption that under this condition Xyr1-sites are occupied, which is supported by *in vivo* footprinting results obtained during this study. We would suggest that Xyr1 recruits chromatin remodelers leading to the observed, more open chromatin status. This provides easier access for the transcription machinery leading to increased induction of the target gene (i.e. the cellulase-encoding gene) under this condition.

In the case of the xylanase-encoding genes we also detected a condition-dependent induction of gene expression, which was accompanied by chromatin opening in the wild-type strain. However, the involvement of Xyr1 is different in this case as compared to the cellulase-encoding genes. In the absence of Xyr1 gene expression decreased under all conditions, but the chromatin in the upstream regions of the xylanase-encoding genes did not always became more compact. For example, in the case of *xyn2,* the URR had a similar chromatin accessibility under non-repressing conditions (sophorose, D-xylose) in the Δ*xyr1*-strain as in the wild-type strain but the gene expression was strongly repressed in the absence of Xyr1. The fact that gene expression can be repressed simultaneously with enhanced chromatin accessibility might be explained by a generally better access for all kinds of regulatory proteins including repressor proteins. Another possible explanation would be that the absence of Xyr1 simply overrules the level of regulation by chromatin opening. Anyway, during this study it became obvious that the activating function of Xyr1 on xylanase-encoding gene expression is not mainly exerted on the chromatin level. There are earlier reports on generally different, condition-dependent transcript level patterns of the *xyn1* and *xyn2* genes as compared to the *xyr1* gene [12]. One example is the low basal *xyn2* gene expression on D-glucose (e.g. [26, 27]) that is not detectable for the *xyr1* gene [13]. All these findings together strongly indicate that additional regulatory factors (for example the suggested xylanase repressor Xpp1 [28]) and mechanisms, which are responsible for chromatin opening under inducing conditions, need to be involved.

A whole transcriptome analysis was used to identify genes classified as chromatin remodelers in *T. reesei*, which are differentially expressed dependent on the

applied condition (inducing/repressing). Notably, 15 genes are differentially expressed in the wild-type strain (compare Table 1), whereas only ten genes responded in a condition-dependent manner in the Δ*xyr1*-strain (data not shown). This again supports the assumption that Xyr1 is generally involved in chromatin remodelling mechanisms. The identification of two putative chromatin remodelers, which are under the control of Xyr1 (directly or via expression of other regulatory proteins), point to an indirect role of Xyr1 in chromatin remodelling. Moreover, it can be speculated that Xyr1 additionally recruits chromatin-remodelling proteins in a differential manner towards the promoters of the cellulase- and xylanase-encoding genes. This would be a further explanation for the observed differences concerning the influence of Xyr1 on their chromatin status. However, at this stage it remains to be investigated if the open chromatin is indeed the result of chromatin remodelling (as the loss or movement of nucleosomes) or if the loss of the identified putative chromatin remodelers overrules the action of Xyr1.

Conclusions

Investigations on the level of chromatin packaging revealed that the transcription factor Xyr1 does exert its activating function—in addition to other possible mechanisms - by an induction-specific opening of chromatin. The impact of Xyr1 in chromatin opening was more pronounced in the case of cellulase-encoding genes than in the case of the xylanase-encoding genes. The application of WTSS identified one chromatin remodeler that is down-regulated under inducing conditions and up-regulated if Xyr1 is missing. According to the results of the present study, this is a target in engineering strains with enhanced cellulase expression.

Methods

Fungal strains

The following *T. reesei* strains were used throughout this study: the wild-type strain QM6a (ATCC 13631), and a corresponding *xyr1* deletion strain (this study), QM9414 (ATCC 26921), and a QM9414 strain bearing a *xyr1* deletion [11]. All strains were maintained on malt extract agar.

Growth conditions

For carbon source replacement experiments mycelia were pre-cultured in 1-L-Erlenmeyer flasks on a rotary shaker (180 rpm) at 30 °C for 24 h in 250 mL of Mandels-Andreotti (MA) medium [29] supplemented with 1 % (w/v) glycerol as sole carbon source. A total of 10^9 conidia per litre (final concentration) were used as inoculum. Pre-grown mycelia were washed and equal amounts were resuspended in 20 ml MA media containing 1 % (w/v) D-glucose or 2 mM sophorose as sole carbon source or no carbon source and were incubated for 3 h.

For direct cultivation experiments conidia were incubated in 200 mL MA medium containing 2 % (w/v) glucose as the sole carbon source for 24 and 48 h. Samples were derived from three biological replicates.

Deletion of *xyr1* from the genome of the *T. reesei* wild-type strain

The deletion of the *xyr1* gene was essentially performed as described in [11]. The plasmid pD2xlr1 was modified by shortening the promoter of the *A. nidulans amdS* gene, which was used as a marker [30]. The obtained plasmid pD5 was applied in a fungal protoplast transformation using QM6aΔ*tmus53* [31] as a recipient strain and was performed by following the protocol described in [32].

CHART-PCR

DNase I digestion of chromatin and DNA extraction were carried out as described before [20]. qPCR analysis of the DNase I-treated samples was performed to measure the relative abundance of target regions. PCRs were performed in triplicates in a Rotor-Gene Q system (Qiagen, Hilden, Germany) using the amplification mixture (final volume 20 μL) and cycling conditions described before [20]. Primer sequences are provided in Table 2. The amount of intact input DNA of each sample was calculated by comparing the threshold values of the PCR amplification plots with a standard curve generated for each primer set using serial dilutions of genomic, undigested DNA. The chromatin accessibility index (CAI) was defined as: $CAI = 1/(Ds/((Dc1 + Dc2)/2))$, where Ds is the amount of intact DNA detected for each target region and Dc1 and Dc2 are the amounts of intact DNA detect for the promoter regions of *sar1* and *act* respectively, used as reference genes for normalization.

Analysis of transcript levels

Fungal mycelia were homogenized in 1 mL of peqGOLD-TriFast DNA/RNA/protein purification system reagent (PEQLAB Biotechnologie, Erlangen, Germany) using a FastPrep(R)-24 cell disrupter (MP Biomedicals, Santa Ana, CA, USA). RNA was isolated according to the manufacturer's instructions, and the concentration was measured using the NanoDrop 1000 (Thermo Scientific, Waltham, US). Synthesis of cDNA from mRNA was carried out using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative PCRs were performed in triplicates in a Rotor-Gene Q system (Qiagen). The amplification mixture (final volume 15 μL) contained 7.5 μL 2 × iQ SYBR Green Mix (Bio-Rad, Hercules, USA),

Table 2 Oligonucleotides used in this study

Name	Sequence (5′ - 3′)	Usage
RG53	GAATTCAGATC	ivFP, oligo-short
RG54	GCGGTGACCCGGGAGATCTGAATTC	ivFP, oligo-long
RG83	[6-FAM]CCTTTGGGTGTACATGTTTGTGCTCCGG	ivFP, cbh1oligo3fw
RG84	[6-FAM]GGAGAGTGCAGGCCGACTGAGC	ivFP, cbh1oligo3rev
RG89	[6-FAM]GTAGAGGCATGTTGTGAATCTGTGTCGGG	ivFP, cbh1oligo3fw
RG90	[6-FAM]GGTTGTATGCAAAACGCTCCGAGTCAGAC	ivFP, cbh1oligo3rev
actfw	TGAGAGCGGTGGTATCCACG	qPCR
actrev	GGTACCACCAGACATGACAATGTTG	
sar1fw	TGGATCGTCAACTGGTTCTACGA	
sar1rev	GCATGTGTAGCAACGTGGTCTTT	
cbh1f	GATGATGACTACGCCAACATGCTG	
cbh1r	ACGGCACCGGGTGTGG	
cbh2f	CTATGCCGGACAGTTTGTGGTG	
cbh2r	GTCAGGCTCAATAACCAGGAGG	
xyn1f	CAGCTATTCGCCTTCCAACAC	
xyn1r	CAAAGTTGATGGGAGCAGAAG	
taqxyn2f	GGTCCAACTCGGGCAACTTT	
taqxyn2r	CCGAGAAGTTGATGACCTTGTTC	
epiactinTr_f	CTTCCCTCCTTTCCTCCCCCTCCAC	act CHART, region −226 to +24
epiactinTr_r	GCGACAGGTGCACGTACCCTCCATT	
episar1Tr_f	GTCAGGAAATGCCGCACAAGCAAGA	sar1 CHART, region −490 to −224
episar1Tr_r	TGTGTTTTACCGCCTTGGCCTTTGG	
epicbh1_1Tr_f	AAGGGAAACCACCGATAGCAGTGTC	cbh1 CHART, region −902 to −610
epicbh1_1Tr_r	TTTCACTTCACCGGAACAAACAAGC	
epicbh1_2Tr_f	GGATCGAACACACTGCTGCCTTTAC	cbh1 CHART, region −301 to −27
epicbh1_2Tr_r	GGTTTCTGTGCCTCAAAAGATGGTG	
epicbh2_1Tr_f	CGGATCTAGGGCAGACTGGGCATTG	cbh2 CHART, region −587 to −338
epicbh2_1Tr_r	GTGTAGTGTTGCGCTGCACCCTGAG	
epicbh2_2Tr_f	TGCAGCGCAACACTACACGCAACAT	cbh2 CHART, region −355 to −62
epicbh2_2Tr_r	TGCGCCTCATACAGGGTCACAGTCC	
epixyn1_1Tr_f	GCACTCCAAGGCCTTCTCCTGTACT	xyn1 CHART, region −577 to −278
epixyn1_1Tr_r	TAGATTGAACGCCACCCGCAATATC	
epixyn1_3Tr_f	GTCGATATTGCGGGTGGCGTTCAAT	xyn1 CHART, region −306 to −10
epixyn1_3Tr_r	TTTGTGCGTGTTTTCCTTGAAGTCG	
epixyn2_1Tr_f	GTGCCGATGAGACGCTGCTGAGAAA	xyn2 CHART, region −527 to −252
epixyn2_1Tr_r	GATATTGCGCCTTGCAACACCATCG	
epixyn2_2Tr_f	CTCGAGACGGCTGAGACAGCAGCAT	xyn2 CHART, region −311 to −38
epixyn2_2Tr_r	TGTCTTTTGGGCTTGGAGGGGTTGT	

100 nM forward and reverse primer and 2.5 μL cDNA (diluted 1:20). Primer sequences are provided in Table 2. Cycling conditions and control reactions were performed as described previously [33]. Data normalization using *sar1* and *act* as reference genes and calculations were performed as published previously [33].

In vivo footprinting

In vivo methylation using DMS followed by ligation mediated PCR was performed as described previously [34]. FAM-labelled fragments were generated by a PCR reaction using RG89 and RG90 or RG83 and RG84 for an URR or a TATA-box containing core region within the

cbh1 promoter, respectively. Primer sequences are provided in Table 2. FAM-labelled fragments were analyzed by capillary gel electrophoresis (Microsynth, Balgach, Switzerland) and results were analyzed using the program ivFAST [34].

Whole transcriptome shotgun sequencing

The mRNA was extracted from fungal mycelia using TRIzol® RNA Kit (Life Technologies, part of Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry at 260/280 nm and RNA integrity was tested by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and gel electrophoresis (1 % agarose). The RNA of the biological replicates was pooled, lyophilized, and stored using the RNAstable® Tube Kit (Biomatrica, San Diego, CA, USA). Barcoded libraries were prepared using the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA, USA) and sequenced by LGC Genomics GmbH (Berlin, Germany) using the Illumina HiSeq 2000 platform.

WTSS data analysis

Sequences from approximately 144 million 100 bp paired-end reads were quality-filtered and mapped to the *Trichoderma reesei* 2.0 reference genome, (http://genome.jgi-psf.org/Trire2/Trire2.home.html) using the Bowtie aligner version 0.12.8 [35] allowing two mismatches and only unique alignments. The SAMtools version 0.1.18 [36] was used to process the alignments files, which were visualized using the Integrative Genomics Viewer [37]. Bioconductor DESeq package version 1.10.1 [38] was utilized for normalization, using the median log deviation, and for the differential expression analysis, applying a two-fold change cut-off, i.e. $\log_2$-fold change ≥ 1 or ≤ -1 and an adjusted p-value ≤ 0.05 were used as thresholds. The $\log_2$-fold change was calculated according to the equation:

$$\log_2\text{-fold change} = \log_2 \frac{baseMeanB}{baseMeanA}, \text{where :}$$

baseMeanB is the mean normalized counts from condition B and baseMeanA is the mean normalized counts from condition A.

Abbreviations

CAI: Chromatin accessibility index; CCR: Carbon catabolite repression; CHART-PCR: Chromatin accessibility real-time PCR; Cre1: Carbon catabolite repressor 1; DMS: Dimethyl sulphate; ivFP: *in vivo* footprinting; KOG: Eukaryotic orthologous groups; MA: Mandels-Andreotti; PCWD: Plant cell wall-degrading/plant cell wall degradation; qPCR: Quantitative PCR; URR: Upstream regulatory region; WTSS: Whole transcriptome shotgun sequencing; Xyr1: Xylanase regulator 1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TMMS performed CHART-PCR and transcript analyses and participated in analysis of WTSS data. AR carried out the *in vivo* footprinting analyses. MEP constructed the *xyr1* deletion strain in QM6a. LSC prepared samples for WTSS and participated in analysis of corresponding data. GFP and RSR participated in analysis of WTSS data. RLM and MJPF participated in conception and revision of the manuscript. RNS participated in design of the study and supervision of experiments. ARMA participated in design of study, supervision of experiments, and prepared the manuscript. All authors read and approved the final manuscript.

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The Relation Between Promoter Chromatin Status, Xyr1 and Cellulase Expression in *Trichoderma reesei*

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Abstract: The ascomycete *Trichoderma reesei* is used for the production of plant cell wall-degrading enzymes in industrial scale. The interplay of the transactivator Xyr1 and the repressor Cre1 mainly regulates the expression of these enzymes. During inducing conditions, such as in the presence of sophorose, the transcription of the two major cellulase-encoding genes, *cbh1* and *cbh2*, is activated as well as the expression of *xyr1*. In the presence of D-glucose carbon catabolite repression mediated by Cre1 takes place and the expression of Xyr1 and the plant cell wall-degrading enzymes is down-regulated. In this study we compare the chromatin status of *xyr1*, *cbh1*, and *cbh2* promoters in the wild-type strain and the Cre1-deficient strain Rut-C30. Chromatin rearrangement occurs in the *xyr1* promoter during induction on sophorose. Chromatin opening and protein-DNA interactions in the *xyr1* promoter were detected especially in a region located 0.9 kb upstream the translation start codon, which bears several putative Cre1-binding sites and a CCAAT-box. Moreover, the *xyr1* promoter is overall more accessible in a *cre1*-truncated background, no matter which carbon source is present. This makes the *xyr1* regulatory sequence a good target for promoter engineering aiming at the enhancement of cellulase production.

Keywords: Cellulases, Chromatin, Promoter, *Trichoderma reesei*, Rut-C30, Xyr1.

1. INTRODUCTION

The saprophytic ascomycete *Trichoderma reesei* (teleomorph *Hypocrea jecorina* [1]) is an industrially relevant microorganism due to its high capacity to secrete plant cell wall-degrading enzymes (PCWDE). These enzymes are applied in the food and textile, pulp and paper industry, and are a bottleneck for the cost-efficient production of second-generation biofuels. Details on applications of *T. reesei* enzymes are reviewed in [2, 3].

The expression of the PCWDE-encoding genes in *T. reesei* is controlled in large part by two transcriptions factors, *i.e.* the main transactivator Xyr1 and the mediator of carbon catabolite repression (CCR), Cre1. Xyr1 is a Zn_2Cys_6 binuclear cluster protein essential for expression of both, xylanases and cellulases [4]. It normally binds to 5'-GGC(A/T)$_3$-3'-motifs [5] arranged as inverted repeats in the promoters regions of its target genes. On most carbon sources *xyr1* is expressed at low levels, however, this can be induced by the disaccharide sophorose [6]. Within the Xyr1 regulon, the induction of the genes coding for the main cellulases (*i.e. cbh1* and *cbh2*) is directly associated with the extent of induction of *xyr1* expression, which is not observed for the other target genes of Xyr1 [6].

Cre1 is a C_2H_2-type zinc finger protein that binds to the consensus sequence 5′-SYGGRG-3′ [7]. In the presence of D-glucose, Cre1 mediates CCR in a double-lock mechanism by directly repressing the expression of some PCWDE-encoding genes [8, 9], and also the expression of *xyr1* itself [10]. It should be noted that the deletion of *cre1* in order to circumvent CCR is not a useful strategy. This deletion causes strong pleiotropic effects, *i.e.* a *cre1* deletion strain is strongly reduced in growth and therefore not convenient for industrial applications.

However, a strain suitable for industrial applications was derived from the *T. reesei* wild-type isolate QM6a by three rounds of mutagenesis. This strain, Rut-C30, was selected for high cellulase production and turned out to be released from Cre1-mediated CCR [11-13]. In total, Rut-C30 lacks over 100 kb of genomic DNA compared to the wild-type strain. In detail, it bears 15 small insertions, 223 point mutations, and 18 deletions [14, 15]. For example, an 83 kb large region containing 29 genes is missing in Rut-C30 [16, 17]. An important feature of Rut-C30 is the presence of a truncated form of the Carbon catabolite repressor 1 (Cre1-96, [7]). Cre1-96 contains only one of the two zinc finger motifs [18], however, it is still able to bind DNA [19]. This truncation does not only result in release from CCR, but additionally has a positive regulatory influence on the expression of target genes [19]. Notably, most industrially used strains originate from Rut-C30 [6, 14].

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Despite the intense efforts to investigate the molecular mechanism underlying the regulation of cellulase production in *T. reesei* (reviewed in [20]), the mechanism of activation of gene expression is still not fully understood. Especially, information about the activation of expression of the *xyr1* gene is scarce. The same is true for possible additional levels of gene regulation within the Xyr1 regulon, such as chromatin remodeling. The upstream regulatory regions (URR) of the cellulase-encoding genes *cbh1* and *cbh2* have been previously subjects in studies focusing on nucleosome positioning and chromatin remodeling in response to different carbon sources. Former reports pointed towards Cre1 as an important factor in influencing chromatin packaging during repressing conditions [19, 21, 22]. During this study we investigated the chromatin accessibility status of the *xyr1, cbh1,* and *cbh2* promoter under repressing and inducing conditions. We aimed to learn more about the *xyr1* promoter architecture and the impact on the cellulase expression in a comparative study including Rut-C30 and the wild-type strain.

2. MATERIALS AND METHODS

2.1. Fungal Strains

The following *T. reesei* strains were used throughout this study: the wild-type strain QM6a (ATCC 13631) and the carbon catabolite derepressed strain Rut-C30 (ATCC 56765). Both strains were maintained on malt extract agar.

2.2. Growth Conditions

For carbon source replacement experiments mycelia were pre-cultured in 1-l Erlenmeyer flasks on a rotary shaker (180 rpm) at 30 °C for 24 h in 250 ml of Mandels-Andreotti (MA) medium [23] supplemented with 1 % (w/v) glycerol as sole carbon source. A total of 10^9 conidia per litre (final concentration) were used as inoculum. Pre-grown mycelia were washed and equal amounts were resuspended in 20 ml MA media containing 1 % (w/v) D-glucose or 2 mM sophorose or no carbon source, respectively, and were incubated for 3 h. Samples were derived from three biological replicates and were pooled before RNA extraction and chromatin digestion.

2.3. Analysis of Transcript Levels

Fungal mycelia were homogenized in 1 ml of peqGOLDTriFast DNA/RNA/protein purification system reagent (PEQLAB Biotechnologie, Erlangen, Germany) using a FastPrep(R)-24 cell disrupter (MP Biomedicals, Santa Ana, CA, USA). RNA was isolated according to the manufacturer's instructions, and the concentration was measured using the NanoDrop 1000 (Thermo Scientific, Waltham, US). Synthesis of cDNA from mRNA was carried out using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Quantitative, reverse transcription PCRs (RT-qPCRs) were performed in a Rotor-Gene Q system (Qiagen, Hilden, Germany). All reactions were performed in triplicates. The reaction mixture (final volume 15 µl) contained 7.5 µl 2 x iQ SYBR Green Mix (Bio-Rad, Hercules, USA), 100 nM forward and reverse primer and 2.5 µl cDNA (diluted 1:20). Primer sequences are provided in (Table **1**). Cycling conditions and control reactions were performed as described previously [24]. Data normalization using *sar1* and *act* as reference genes and calculations were performed as published previously [24].

2.4. Chromatin Accessibility Real-time PCR (CHART-PCR)

DNase I digestion of chromatin and quantitative PCR (qPCR) analysis were carried out as described before [19]. qPCR analysis of the DNase I-treated samples was performed to measure the relative abundance of target regions. PCRs were performed in triplicates in a Rotor-Gene Q system (Qiagen) using the reaction mixture (final volume 20 µl) and cycling conditions described before [19]. Primer sequences are provided in (Table **1**). The amount of intact input DNA of each sample was calculated by comparing the threshold values of the PCR amplification plots with a standard curve generated for each primer set using serial dilutions of genomic, undigested DNA. The chromatin accessibility index (CAI) was defined as: CAI = (Dc1+Dc2)/2Ds, where Ds is the amount of intact DNA detected for each target region and Dc1 and Dc2 are the amounts of intact DNA detected for the promoter regions of *sar1* and *act* respectively, used as reference genes for normalization.

2.5. *In Vivo* Footprinting Analysis

In vivo methylation using dimethyl sulphate (DMS) followed by ligation-mediated PCR was performed as described previously [25]. Primer sequences are provided in (Table **1**). The separation of fragments was performed by capillary gel electrophoresis (Microsynth, Balgach, Switzerland) and results were analyzed using the ivFAST program [25]. For the generation of the landscape visualization, the data sets of the coding and non-coding strand were combined, for each base pair the mean of the output value of this base pair and of the four adjacent base pairs was calculated, obtained mean values were converted to logarithmic scale, and given as the protein-DNA interaction index (PDI). In the case of no difference between compared conditions, termed 'n/a' in the ivFAST data output file, the value was set to 0.

3. RESULTS

3.1. Higher *xyr1* Expression Corresponds to Higher Chromatin Accessibility in the *xyr1* Promoter

We were interested to what extent chromatin accessibility of the *xyr1* promoter is linked to the transcript levels of *xyr1*. Therefore, we subjected the wild-type strain QM6a and Rut-C30 to a carbon source replacement experiment. After pregrowing both strains in MA medium containing glycerol, the obtained mycelia were transferred to MA medium containing sophorose (inducing condition), D-glucose (repressing condition) or no carbon source (reference condition) and were incubated for 3 h. First, CHART-PCR was employed to investigate the chromatin packaging of two *xyr1* promoter regions: the core promoter (position -216 to +35) bearing the TATA-box, and a putative URR (position -1038 to -742) bearing five Cre1-binding sites (positions -872, -878, -945, -963, and -972) and a CCAAT-box (-995, [26]) (Fig. **1A**). Complementary, the expression of *xyr1* was investigated by RT-qPCR in order to evaluate its relation to chromatin accessibility. The expression of *xyr1* is equally induced on sophorose in the two strains, whereas D-glucose represses

Table 1. Oligonucleotides used throughout this study.

Name	Sequence (5' -3')	Usage
Actfw	TGAGAGCGGTGGTATCCACG	RT-qPCR
Actrev	GGTACCACCAGACATGACAATGTTG	RT-qPCR
sar1fw	TGGATCGTCAACTGGTTCTACGA	RT-qPCR
sar1rev	GCATGTGTAGCAACGTGGTCTTT	RT-qPCR
cbh1f	GATGATGACTACGCCAACATGCTG	RT-qPCR
cbh1r	ACGGCACCGGGTGTGG	RT-qPCR
cbh2f	CTATGCCGGACAGTTTGTGGTG	RT-qPCR
cbh2r	GTCAGGCTCAATAACCAGGAGG	RT-qPCR
xyr1f	CCCATTCGGCGGAGGATCAG	RT-qPCR
xyr1r	CGAATTCTATACAATGGGCACATGGG	RT-qPCR
epiactinTr_f	CTTCCCTCCTTTCCTCCCCCTCCAC	*act* CHART, region -226 to +24
epiactinTr_r	GCGACAGGTGCACGTACCCTCCATT	*act* CHART, region -226 to +24
episar1Tr_f	GTCAGGAAATGCCGCACAAGCAAGA	*sar1* CHART, region -490 to -224
episar1Tr_r	TGTGTTTTACCGCCTTGGCCTTTGG	*sar1* CHART, region -490 to -224
epixyr1_1Tr_f	CCTTTGGCCATCTACACAAGAGCAA	*xyr1* CHART, region -1038 to -742
epixyr1_1Tr_r	CGCAATTTTTATTGCTGTTCGCTTC	*xyr1* CHART, region -1038 to -742
epixyr1_2Tr_f	CCGACAGCAGCAGTAGTCAGGTTTT	*xyr1* CHART, region -216 to +35
epixyr1_2Tr_r	TAGGCAGAATAGCGACGGAGAGGAT	*xyr1* CHART, region -216 to +35
epicbh1_1Tr_f	AAGGGAAACCACCGATAGCAGTGTC	*cbh1* CHART, region -902 to -610
epicbh1_1Tr_r	TTTCACTTCACCGGAACAAACAAGC	*cbh1* CHART, region -902 to -610
epicbh1_2Tr_f	GGATCGAACACACTGCTGCCTTTAC	*cbh1* CHART, region -301 to -27
epicbh1_2Tr_r	GGTTTCTGTGCCTCAAAAGATGGTG	*cbh1* CHART, region -301 to -27
epicbh2_1Tr_f	CGGATCTAGGGCAGACTGGGCATTG	*cbh2* CHART, region -587 to -338
epicbh2_1Tr_r	GTGTAGTGTTGCGCTGCACCCTGAG	*cbh2* CHART, region -587 to -338
epicbh2_2Tr_f	TGCAGCGCAACACTACACGCAACAT	*cbh2* CHART, region -355 to -62
epicbh2_2Tr_r	TGCGCCTCATACAGGGTCACAGTCC	*cbh2* CHART, region -355 to -62
RG161	[6-FAM]AGGAGGAGCACGAACTGGACCGCAA	*xyr1* URR iv fp, fwd
RG162	[6-FAM]GACACGACCAGAGAGCTCCATCATGG	*xyr1* URR iv fp, rev
RG149	[6-FAM]CCTGCTCAGAGCTTGGCGCGATTTC	*xyr1* core region iv fp, fwd
RG150	[6-FAM]TCGGGGTAGGCAGAATAGCGACGGA	*xyr1* core region iv fp, rev

xyr1 expression only in the wild-type strain (Fig. **1B, C**). In both strains, the induced *xyr1* transcript formation on sophorose went along with a more open chromatin status compared to D-glucose. This could be found in the investigated URR (Fig. **1B**) and in the core promoter (Fig. **1C**). However, a comparison of the strains under the same condition (*i.e.* D-glucose repression or sophorose-mediated induction) revealed that the chromatin is in all cases more accessible in Rut-C30 (Fig. **1B, C**). This indicates a generally more accessible *xyr1* promoter in Rut-C30 than in the wild-type strain.

3.2. A Condition-specific Predisposition for DNA Occupancy in the URR of the *xyr1* Promoter is Lost in Rut-C30

The observed, strain-specific differences in chromatin compaction of the *xyr1* promoter prompted us to perform a detailed investigation of the DNA accessibility. To this end,

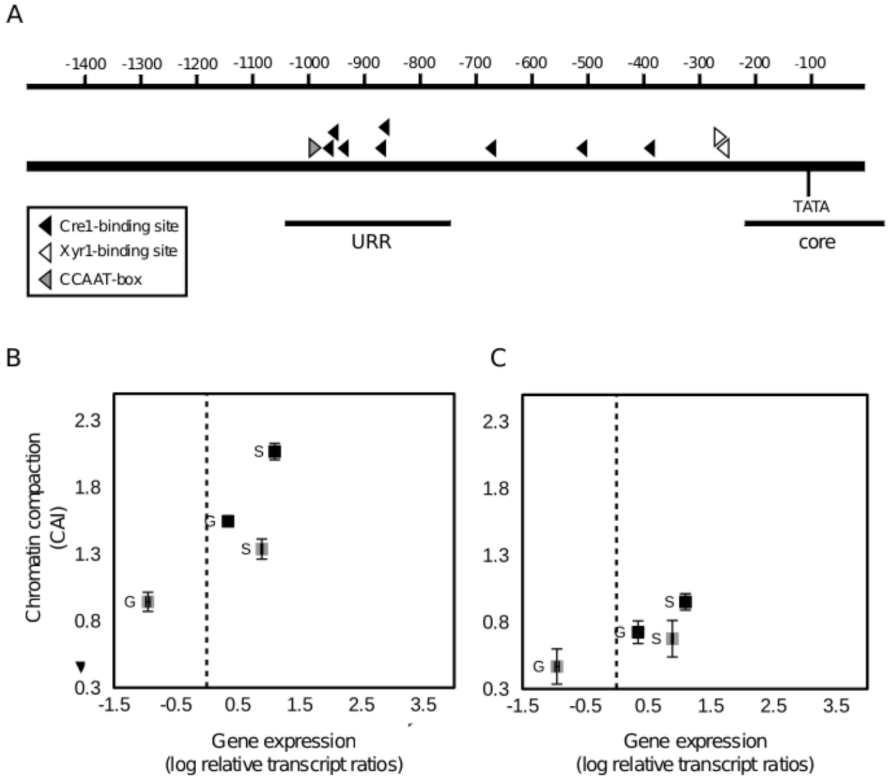

Fig. (1). Chromatin status and gene expression of *xyr1* in the wild-type strain and Rut-C30. (**A**) The promoter of *xyr1* is schematically represented. The scale at the top indicates the distance from the start codon in bp. Black bars indicate the positions of the core region (core) and the URR (URR) investigated by CHART-PCR. Triangles indicate *cis* elements. For CHART-PCR of the URR (**B**) and the core promoter (**C**) and *xyr1* transcript analysis (**B, C**) the *T. reesei* wild-type strain (grey squares) and Rut-C30 (black squares) were pre-grown on glycerol and thereafter incubated on D-glucose (G) or sophorose (S) for 3 h. CHART-PCR was performed by DNase I digestion followed by qPCR, and chromatin accessibility indices (CAIs) are depicted on the y-axis. The gene expression analysis was performed by cDNA synthesis followed by qPCR, and transcript levels are depicted on the x-axis. The dashed line indicates transcript level of the reference condition (wild-type strain, no carbon source, 3 h). *sar1* and *act* genes were used for data normalization. All values are means from measurements in triplicates and three biological replicates. Error bars indicate standard deviations. Diagrams are identically scaled.

in vivo footprinting analyses of an URR and the core promoter region were performed to identify differences in protein-DNA interaction patterns. Again, both strains were subjected to a carbon source replacement experiment as described above, followed by DMS-induced *in vivo* methylation. We could detect high DNA occupancy signals in the vicinity of the TATA-box, indicating a highly active transcription of *xyr1* in both strains during sophorose induction (Fig. **2B**). This is in good accordance with the before observed sophorose-mediated induction of transcript formation (compare Fig. **1B, C**). However, an overall analysis of the two regions resulted in different profiles for the two strains. Rut-C30 exhibited a repetitive signal appearance along the two analyzed regions, indicating widespread differences in DNA accessibility on sophorose and D-glucose (Fig. **2A, B**). In contrary, in the wild-type strain, the footprinting patterns turned out to differ between the two investigated *xyr1* promoter regions. For the URR we detected a high number of strong signals indicating considerable increased protein-DNA interaction under inducing condition compared to repressing condition (Fig. **2A**) while less and weaker signals were detected in the core promoter (Fig. **2B**). Altogether, these data support the assumption of a generally higher chromatin accessibility within the *xyr1* promoter in Rut-C30. It should be noted that we detected more and higher signals in the URR of QM6a than in the one of Rut-C30 (Fig. **2A**). The increased chromatin accessibility in the URR of the wild-type strain might be necessary to result in the same high transcript levels of *xyr1* during sophorose induction as in Rut-C30. Finally, we found that a single Xyr1-binding site (5'-GGCTAT-3', position -265 on the non-coding strand) is occupied in both strains, with stronger signals for Rut-C30 (Fig. **2C**). A second, non-canonical Xyr1-binding site (5'-GGGTTA-3', position – 270 on the coding strand) close to the first one was detected.

3.3. Expression of Cellulase-encoding Genes is Not Directly Influenced By the Promoter Chromatin Status During Early Induction

We observed a higher accessible chromatin status in the *xyr1* promoter in Rut-C30 compared to the wild-type strain. To examine whether this is also present in the targets genes of Xyr1, we comparatively investigated the chromatin status of the core promoter and an URR of the *cbh1* and *cbh2* genes each. Complementary, we analyzed their transcript levels. In contrast to what was observed for *xyr1*, no remarkable differences in the chromatin status of the *cbh1* (Fig. **3C, D**) nor

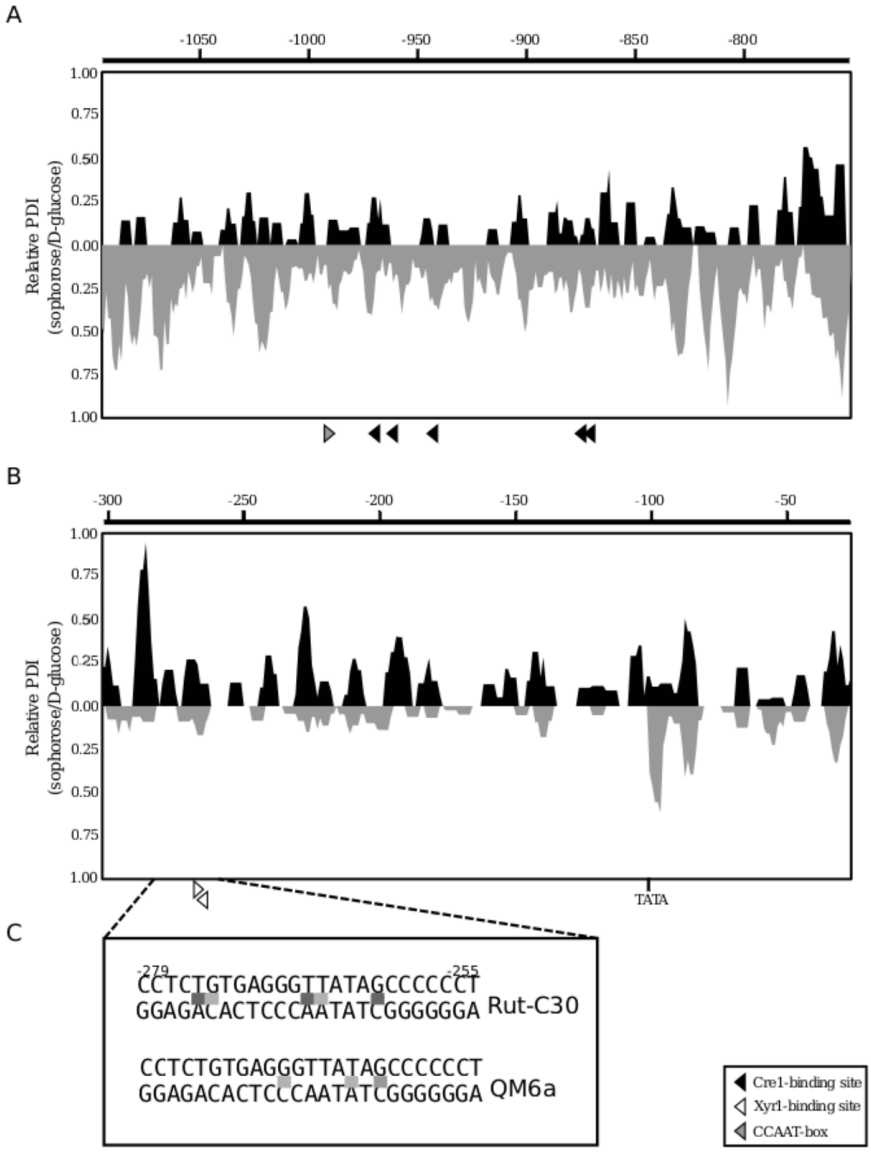

Fig. (2). *In vivo* foot printing analysis of the *xyr1* promoter. The *T. reesei* wild-type strain (grey landscape) and Rut-C30 (black landscape) were pre-grown on glycerol and then incubated on D-glucose or sophorose for 3 h followed by DMS-induced *in vivo* methylation. An URR (**A**) and the core promoter (**B**) were investigated. Triangles indicate *cis* elements. Analysis of data was performed using ivFAST [25] followed by a landscape-like visualization. The relative protein-DNA-interaction indices (PDI) give differences between the *in vivo* footprinting patterns obtained on sophorose compared to D-glucose of one strain. The diagrams are identically scaled and are comparable amongst the two strains within one region. The scale at the top indicates distance from the start codon in bp. (**C**) Detailed *in vivo* footprint result for both strains of the region -279 to -255 bearing two overlapping Xyr1-binding sites (bold letters). Methylation differences between sophorose and D-glucose are represented by light grey (1.1- to 1.3-fold) and dark grey squares (more than 1.3-fold).

of the *cbh2* (Fig. **3E, F**) promoters could be detected between the two strains. Besides this, we detected a slight sophorose-specific chromatin opening for each strain compared to D-glucose (Fig. **3C-F**). As expected, expression of both genes on D-glucose was partly released from CCR in Rut-C30. Transcript levels of *cbh1* and *cbh2* under inducing conditions were similar in both strains (Fig. **3C** and **E**), which fits to the before observed equal *xyr1* transcript levels (compare Fig. **1B**). Even if the above described results point to a relation between the chromatin status of the promoter and transcript formation in case of the *xyr1* gene, the expression of the *cbh1* and *cbh2* genes during early induction seem to be rather a consequence of the levels of Xyr1 than influenced by the chromatin status of their own promoters.

4. DISCUSSION

It has recently been reported that chromatin remodeling is associated with gene expression of PCWDEs in *T. reesei* in dependence of the carbon source used [19, 21, 22]. Moreover, Xyr1 is absolutely required for activation of PCWDE-encoding genes since a *xyr1* deletion fully abolishes their expression [4, 27]. However, up to now it remained completely unstudied whether chromatin remodeling is also influencing the expression of *xyr1* itself, and thereby,

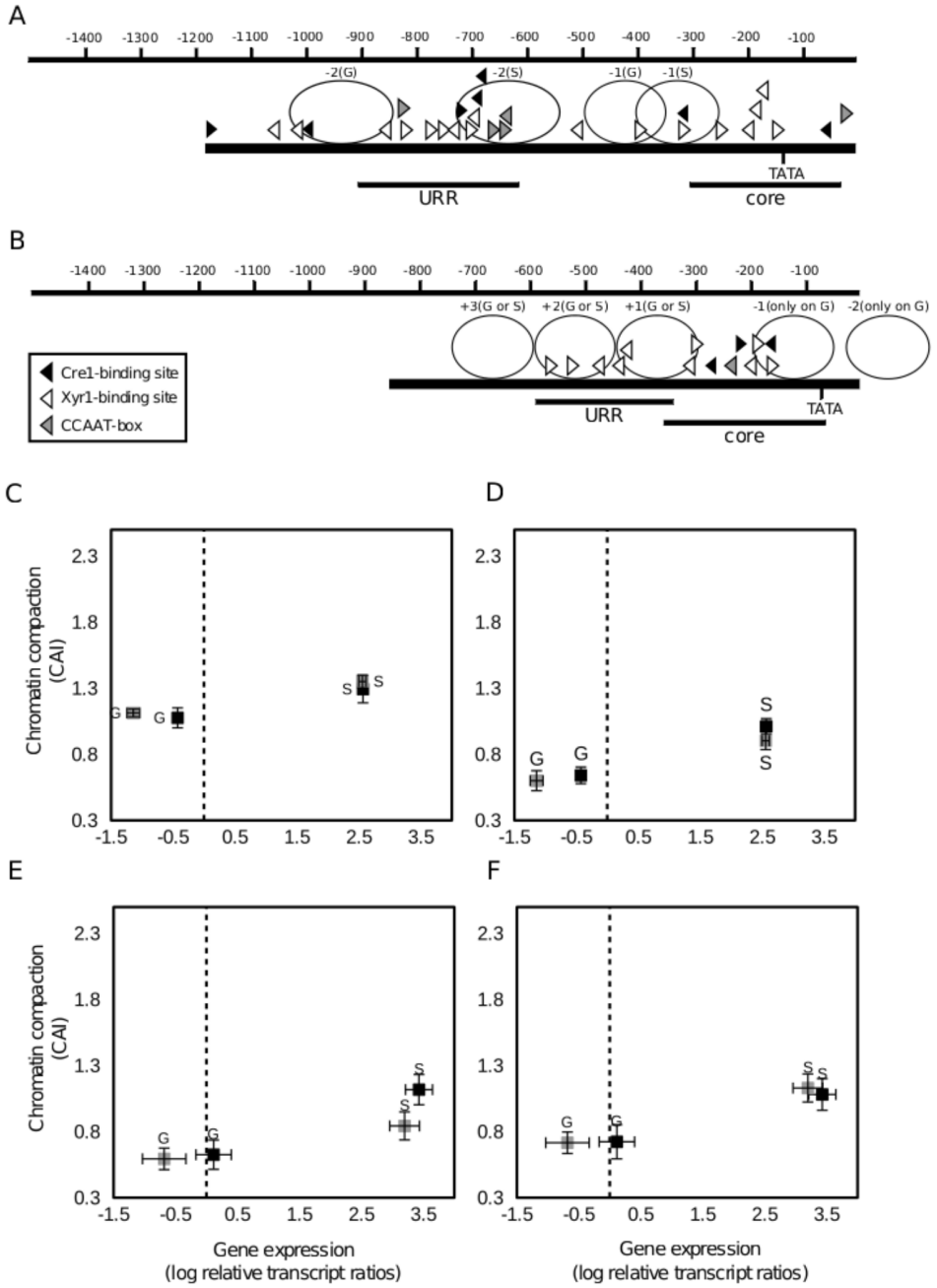

Fig. (3). Chromatin status and gene expression of cellulase-encoding genes in the wild-type strain and Rut-C30. The promoters of *cbh1* (**A**) and *cbh2* (**B**) are schematically represented. The scale at the top indicates the distance from the start codon in bp. Black bars indicate the positions of the core region (core) and the URR (URR) investigated by CHART-PCR. Triangles indicate *cis* elements. Carbon source-specifically positioned nucleosomes [21, 22] are indicated by ellipses. (**C-F**) The *T. reesei* wild-type strain (grey squares) and Rut-C30 (black squares) were pre-grown on glycerol and thereafter incubated on D-glucose (G) or sophorose (S) for 3 h. The URR (**C, E**) and the core promoter region (**D, F**) of the *cbh1* (**C, D**) and *cbh2* (**E, F**) genes were investigated each. CHART-PCR was performed by DNase I digestion followed by qPCR, and chromatin accessibility indices (CAIs) are depicted on the y-axis. The gene expression analysis was performed by cDNA synthesis followed by qPCR, and transcript levels are depicted on the x-axis. The dashed line indicates transcript level of the reference condition (wild-type strain, no carbon source, 3 h). *sar1* and *act* genes were used for data normalization. All values are means from measurements in triplicates and three biological replicates. Error bars indicate standard deviations. Diagrams are identically scaled.

might influence PCWDE-encoding gene expression in an indirect way. We found that induction-specific opening of chromatin is a lot more pronounced in the *xyr1* promoter than in those of the target genes, *cbh1* and *cbh2*. In the case of *xyr1* this chromatin opening goes hand in hand with higher gene expression, while for the target genes *cbh1* and *cbh2* the early induction is not directly related with opening of the chromatin. However, nucleosome positioning in *cbh1* and *cbh2* has previously been studied in *T. reesei* [21, 22]. Comparing the wild-type strain with *cre1*-deficient strains

(including Rut-C30) revealed that in the latter no positioned nucleosomes are present in the coding region of *cbh1* during cultivation on D-glucose. The authors also suggested a repositioning of promoter nucleosomes during inducing condition. Another study reported that two nucleosomes downstream of an *cbh2* activating element lose their positioning in a Cre1 positive background during induction, whereas Rut-C30 constitutively lacked strictly positioned nucleosomes on the *cbh2* promoter under all conditions tested [22]. Both studies emphasize a possible role of Cre1 in organizing the local chromatin structure in *cbh1* and *cbh2* during repressing conditions. It was speculated that a loss of functional Cre1 results in a less dense chromatin structure during CCR. At first glance, the similar chromatin compaction in the wild-type strain and Rut-C30 on D-glucose, which was observed during this study, seem to contradict these earlier studies. However, considering the time frame of the experiments, they turn out to be complementary. The previous nucleosome mapping in *cbh1* was investigated in a time course experiment by direct cultivation of the strains [21], and data for *cbh2* were obtained from longer incubation times after the carbon source replacement [22]. As we investigated the chromatin status already after 3 h, it is likely that chromatin remodeling had only influenced the transactivator-encoding gene expression. This led to enough available Xyr1 that consecutively positively influenced the expression of *cbh1* and *cbh2*. This conclusion is in accordance with the previously reported strict dependence of gene expression of *cbh1* and *cbh2* on the level of *xyr1* [6]. We suppose that the previously observed chromatin remodeling in the promoters of the target genes (*chb1* and *cbh2*) takes place during later cultivation stages. The authors would even suggest that Xyr1 then even contributes to a chromatin opening in the URR of cellulase-encoding genes since recently obtained data support this hypothesis (Mello-de-Sousa *et al*., 2015, unpublished data).

Notably, we found that a *cre1* truncation background contributes to higher *xyr1* expression that goes along with chromatin opening regardless the condition employed. The increased accessibility of chromatin in Rut-C30 was verified by *in vivo* footprinting. During this experiment we detected a canonical Xyr1-binding site next to a non-canonical site in the *xyr1* promoter. It should be mentioned that previous *in vivo* footprinting analyses also detected active non-canonical Xyr1-binding sites (bearing up to two mismatches) in the *cbh2* and *xyn2* promoters [25]. The occuring Xyr1 double binding site (typically arranged as an inverted repeat) in the *xyr1* promoter points towards an eventual autoregulatory mechanism. According to the *in vivo* footprinting analysis this Xyr1-binding motif is stronger contacted Rut-C30. This would allow more available Xyr1 in Rut-C30, which might explain the observed differences between the strains on sophorose.

In the case of D-glucose the strongest differences in chromatin accessibility between Rut-C30 and wild-type strain were observed for the *xyr1* URR. This region bears five putative Cre1-binding sites and a CCAAT-box. The close position of the 5'-SYGGRG-3' motifs to each other and the strong signals detected in the wild-type strain point to an occupation by Cre1. This is a likely explanation for the more compact chromatin status observed in the wild-type strain on D-glucose. The presence of a CCAAT-box reinforces the importance of this region since the interaction of this *cis* element with the HAP2/3/5 complex is required for chromatin remodeling and full transcriptional activation of *cbh2* [22, 28]. *In vivo* footprinting analysis carried out in this study highlighted strong protein-DNA interactions in both strains at this motif during sophorose induction.

5. CONCLUSIONS

The investigation of chromatin accessibility of the *xyr1*, *cbh1,* and *cbh2* promoters revealed that during early induction chromatin rearrangement targets primary the *xyr1* promoter. An upstream regulatory region in the *xyr1* promoter was detected to be an important target of chromatin opening, probably involving regulation by Cre1 interaction and the protein complex HAP2/3/5. Consequently, the *xyr1* promoter represents a high potential target for strain engineering and employing approaches involving heterochromatin control of gene expression.

LIST OF ABBREVIATIONS

CAI	=	Chromatin accessibility index
CCR	=	Carbon catabolite repression
CHART-PCR	=	Chromatin accessibility real-time PCR
Cre1	=	Carbon catabolite repressor 1
DMS	=	Dimethyl sulphate
MA	=	Mandels Andreotti
PCWDEs	=	Plant cell wall-degrading enzymes
PDI	=	Protein-DNA interaction index
qPCR	=	Quantitative PCR
RT-qPCR	=	Quantitative, reverse transcription PCR
URR	=	Upstream regulatory region
Xyr1	=	Xylanase regulator 1

CONFLICT OF INTEREST

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

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TMMS participated in conception of the study, performed CHART-PCR, transcript analyses, and drafted the manuscript. AR participated in conception of the landscape visualization of the *in vivo* footprinting data and carried out the footprinting analyses. CD participated in conception of the landscape visualization of the *in vivo* footprinting data and revision of the manuscript. RLM and MJPF participated in conception of the study and revision of the manuscript. ARMA participated in conception of the study, supervision of experiments, and revision of the manuscript. All authors read and approved the final manuscript. This work was supported by two grants from the Austrian Science Fund (FWF): V232-B20, P24851 given to ARMA, and by a doctoral program of Vienna University of Technology ("CatMat").

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List of Publications

Articles Published in Scientific Journals

1. **Mello-De-Sousa, Thiago Machado;** Rassinger, Alice; Derntl, Christian; Poças-Fonseca, Marcio José; Mach, Robert; Mach-Aigner, Astrid. The Relation between Promoter Chromatin Status, Xyr1 and Cellulase Expression in *Trichoderma Reesei*. Current Genomics. v.17, p.1 - 1, **2016**.
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