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ORIGINAL ARTICLE

Transcriptional analysis of the lichenase-like gene *cel12A* of the filamentous fungus *Stachybotrys atra* BP-A and its relevance for lignocellulose depolymerization

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Abstract

To rationally optimize the production of industrial enzymes by molecular means requires previous knowledge of the regulatory circuits controlling the expression of the corresponding genes. The genus *Stachybotrys* is an outstanding producer of cellulose-degrading enzymes. Previous studies isolated and characterized the lichenase-like/non-typical cellulase Cel12A of *S. atra* (AKA *S. chartarum*) belonging to glycosyl hydrolase family 12 (GH12). In this study, we used RT-qPCR to determine the pattern of expression of *cel12A* under different carbon sources and initial ambient pH. Among the carbon sources examined, rice straw triggered a greater increase in the expression of *cel12A* than 1% lactose or 0.1% glucose, indicating specific induction by rice straw. In contrast, *cel12A* was repressed in the presence of glucose even when combined with this inducer. The proximity of 2 adjacent 5′-CTGGGGTCTGGGG-3′ CreA consensus target sites to the translational start site of *cel12A* strongly suggests that the carbon catabolite repression observed is directly mediated by CreA. Ambient pH did not have a significant effect on *cel12A* expression. These findings present new knowledge on transcriptional regulatory networks in *Stachybotrys* associated with cellulose/hemicellulose depolymerization. Rational engineering of CreA to remove CCR could constitute a novel strategy for improving the production of Cel12A.

Keywords *Stachybotrys* · Lichenase · Transcription regulation · RT-qPCR · Agricultural waste · Biomass depolymerization

Introduction

Filamentous fungi have the ability to produce a plethora of extracellular enzymes enabling them to utilize a wide spectrum of plant cell wall polysaccharides (e.g., cellulose, hemicelluloses, and pectins), and thus they play a key step in the carbon cycle. The ecological and biotechnological importance of fungi and their extracellular plant cell wall-degrading enzymes (PCWDEs), as well as the importance of these enzymes in fungal nutrition and lifestyles, have promoted interest towards understanding the molecular mechanisms controlling their production. As the production of extracellular enzymes in large quantities is an energy-consuming process, it is not surprising that the enzymatic degradation of plant polysaccharides and the subsequent utilization of their components as carbon and energy sources are highly regulated events (at the level of transcription) that ensure the hierarchical and conditional use of these substrates (MacCabe et al. 2002; Aro et al. 2005).

Carbon source-dependent regulation of genes encoding PCWDEs occurs by at least two independent mechanisms: induction in the presence of the polymeric substrate or its degradation products and carbon catabolite repression (CCR) triggered by easily metabolizable carbon sources such as glucose (Martin et al. 2007; Amore et al. 2013; Adnan et al. 2018). Since plant polysaccharides are too large to enter the fungal cell, it is commonly accepted that their capacity to induce the expression of PCWDE genes resides in the production of a basal level of extracellular enzymatic activity that results in the liberation of a soluble inducer (reviewed by Amore et al. 2013). PCWDE gene induction has been studied

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in a number of filamentous fungi. In *Hypocrea jecorina* (*Trichoderma reesei*), *Neurospora crassa*, *Aspergillus niger*, and *Aspergillus nidulans*, the binuclear zinc cluster (Zn2Cys6) transcription factors CLR-1/ClrA (proposed binding site 5′-CGGN$_5$CGGNCCG-3′), CLR-2/ClrB (5′-CGGN$_{11}$CGG-3′ or 5′-YAGAAT-3′), Ace2/AceB (5′-GGCTAATAA-3′ or 5′-GGGTAAATTGG-3′ or 5′-GGCW$_4$-3′), and XlnR/Xyr1/Xlr-1 (5′-GGCTAA-3′, 5′-GGCTRRR-3′, 5′-GGCWWW-3′) activate the expression of genes required for cellulose and/or hemicellulose deconstruction (see Coradetti et al. 2012; Amore et al. 2013; Huberman et al. 2016; Benocci et al. 2017; and references therein), though not all these factors are ubiquitous in these organisms (and others) and the gene sets activated are not necessarily identical.

CCR is a general regulatory mechanism that also controls whether PCWDEs are produced or not. In the presence of preferred carbon sources such as glucose, the expression of genes required for the utilization of alternative carbon sources (e.g., cellulose, hemicellulose) is prevented by the wide-domain Cys2His2-type zinc finger repressor CreA/CRE1/CRE-1 (see Amore et al. 2013; Huberman et al. 2016; Benocci et al. 2017; and references therein). CreA has been extensively characterized in *A. nidulans*, and CreA homologs have been also found in other filamentous fungi. It has been shown that CCR is exercised via binding of CreA to DNA targets with the consensus sequence 5′-SYGGRG-3′ (Kulmburg et al. 1993; Cubero and Scazzocchio 1994).

In addition, though not being carbon source regulation, the enzymatic breakdown of plant cell wall polymers can occur at different environmental pH values, and fungi have also developed regulatory circuits to ensure that the metabolically costly synthesis of PCWDEs does not occur under unfavorable pH conditions where these enzymes are less active. The regulatory mechanism controlling pH-dependent transcriptional regulation has been extensively analyzed in *A. nidulans*, and a major role for the Cys2His2 wide-domain pH regulator PacC (binding site 5′-GCCARG-3′) has been shown (see Peñalva and Arst Jr 2004; Peñalva et al. 2008; and references therein). The first demonstration that the expression of fungal genes encoding extracellular PCWDEs is regulated by environmental pH via PacC was that of the xylanolytic genes *xlnA* and *xlnB* of *A. nidulans* (MacCabe et al. 1998).

The genus *Stachybotrys* (Sordariomycete class) comprises several species, most having been isolated from agricultural wastes and other decomposing cellulosic materials (Abdel-Mallek 1994; Wang et al. 2015). These biomass-degrading fungi thus exhibit efficient enzymatic systems for the degradation of these substrates, including alkaline-resistant and thermostable cellulases, and β-glucanases with potential applications in the paper, textile, food, and biofuel industries (Picart et al. 2016; Picart et al. 2008; Saibi et al. 2007; Taylor et al. 2002; Tweddell et al. 1996). Notwithstanding the biotechnological relevance of *Stachybotrys*, studies on transcriptional regulatory networks controlling the expression of its PCWDE genes are scarce at present. Only a few studies reporting the expression of β-glucosidase genes of *Stachybotrys microspora* (using semi-quantitative RT-PCR assays) have been reported (Abdeljalil et al. 2013, 2014; Ben Hmad et al. 2014). In addition, few studies on the expression of genes involved in the mycoparasitism of *Stachybotrys elegans* have been also published (Morissette et al. 2003, 2006, 2008; Chamoun et al. 2015). Probably, the fact that several strains of the genus *Stachybotrys* have been reported as potentially toxigenic has hampered the study of their biomass-degrading enzymatic system (Etzel et al. 1998; Brasel et al. 2005).

We have previously isolated the strain *S. atra* BP-A from a rotting cellulose rag, and one of the genes encoding a major PCWDE produced by this strain (*cel12A*) has been cloned and characterized (Picart et al. 2012). The product of this gene, Cel12A, belongs to the glycosyl hydrolase family 12 (GH12) and was found to be a lichenase-like or non-typical cellulase showing low activity on cellulose but high activity on lichenan (β-1,3-1,4-glucan) and barley mixed glucans ((1 → 3, 1 → 4)-β-D-glucans). These properties show the potential application of Cel12A in the brewing and wine industries to facilitate filtration processes; in animal foodstuffs to improve β-glucans digestibility and nutritive quality; to produce valuable oligosaccharides; and in the processing of agricultural and industrial wastes for producing bioethanol and biodiesel (Thomas 1956; Goldenkova-Pavlova et al. 2018; Chaari and Ellouz Chaabouni 2019). The aim of the present work was to study (real-time RT-qPCR) the effect of different carbon sources and initial pH values on the expression of *cel12A*. To the best of our knowledge, this is the first report on the regulation of a lichenase gene in the genus *Stachybotrys* and established a possible direct role for the carbon catabolite repressor CreA and its CCR. We have identified (orthology predictions) putative transcription regulators of the PCWDE gene *cel12A* in the genus *Stachybotrys*. We believe that this work could be relevant to study the regulation of other PCWDE genes (e.g., encoding thermostable cellulases), as well as genes associated with mycoparasitism (e.g., *sechi44* encoding a chitinase repressed by glucose; Morisette el al., 2006) in the genus *Stachybotrys*, an outstanding cellulolytic fungus.

Material and methods

Strains and culture conditions

Stachybotrys atra BP-A was previously isolated in our research group and maintained at 30 °C on potato dextrose agar (PDA) as described (Picart et al. 2008). Rice straw was

collected from *Oryza sativa* cultivated fields in La Albufera de Valencia (Spain).

To obtain mycelium for enzyme production and RNA extraction under different growth conditions, 10^6 spores/mL were inoculated in 50 mL of non-buffered basal media (BM; pH 6.5) (Mandels and Weber 1969) supplemented with 1% (w/v) glucose and incubated at 30 °C and 200 rpm. After 24 h, pregrown mycelium was filtered, washed, and transferred to fresh 50 mL BM, supplemented with the following carbon sources: 1% (w/v) glucose, 0.1% (w/v) glucose, 1% (w/v) lactose, 1% (w/v) rice straw, 1% (w/v) rice straw + 1% (w/v) glucose, and 1% (w/v) rice straw + 0.1% (w/v) glucose, and incubated for another 16 h at 30 °C and 200 rpm. Similarly, to achieve the different conditions with respect to ambient pH, pregrown mycelium (BM + 1% glucose) was transferred to induction media (BM + 1% rice straw) buffered to acidic (pH 5.2) or alkaline (pH 7.9) conditions using 100 mM phosphate and 200 mM Na^+ as described (Orejas et al. 1995) and incubated for another 16 h at 30 °C and 200 rpm. After incubation, supernatants were collected for lichenase activity assay and protein quantification, whereas the mycelia were harvested by filtration using Miracloth filters (Calbiochem®, CA, USA) for subsequent RNA extraction. All experiments were done by triplicate.

Lichenase activity assay and protein quantification

Enzyme activity was assayed by measuring the amount of reducing sugars released from lichenan using the method of Nelson and Somogyi (Spiro 1966). The assay mixtures contained 1.5% (w/v) lichenan in a final volume of 0.1 mL of 50 mM phosphate buffer at pH 6. The mixtures were incubated at 45 °C for 15 min. Color development was measured at 520 nm. One unit of lichenase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent per min under the assay conditions described.

The Bradford protein assay was used to measure the concentration of total extracellular proteins (Bradford 1976) using BSA as standard. All assays were performed in triplicate.

RNA extraction and reverse transcription

Harvested mycelia were immediately ground in liquid nitrogen to a fine powder. Total RNAs were extracted by using the FastRNA® kit (Qbiogene) and treated with RNase-free DNase I™ (QIAGEN) according to the manufacturer's recommendations. The RNA concentration and purity (A260/A280 and 260/230 ratios) were determined using a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies), and agarose (1.1% w/v) gel electrophoresis was conducted to visualize the integrity of the RNAs. Only undegraded RNA samples with an A260/A280 ratio between 1.9 and 2.1 and A260/A230 ratios greater than 2.0 were used for the analyses.

First strand cDNAs were synthesized from 1 μg of total RNA using random hexamers as primers and SuperScript™ II (MoMLV-RT, Roche) according to the manufacturer's instructions.

Expression analysis of cel12A by real-time quantitative RT-qPCR

RT-qPCR was performed for the target gene *cel12A* and for the housekeeping reference gene *18S rRNA* using the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems) and the TaqMan® probe (Applied Biosystems), following the manufacturer's instructions. Amplification assays were performed in 30 μL reactions containing the following concentrations: 0.9 mmols of each primer, 0.2 mmols TaqMan® probe, 1 unit of HotSplit DNA polymerase (Biotools), and 10 ng of template cDNA. Primers and TaqMan® probes used are listed in Table 1. The amplification conditions were 95 °C for 10 min (hot start), followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C. Reactions without cDNA functioned as negative controls. All assays were performed in triplicate. Following amplification, the specificity of the PCR products was confirmed by melting curve analyses.

We choose to use RT-qPCR instead northern blotting because it is more convenient for the detection and quantification of relative amounts of mRNA (i.e., it does not require large amounts of RNA), highly sensitive, accurate, and reproducible, thus allowing the examination of *cel12A* expression under different conditions including those resulting in very low levels of expression.

Data quantification

Relative *cel12A* expression analysis was performed by the $2^{-\Delta\Delta Ct}$ method (Morse et al. 2005) and normalized against the expression of *18S rRNA*, which exhibited no significant C_t (threshold cycle) variation in all tested experimental conditions. Real-time RT-qPCR data were calculated as relative expression values (fold change) of *cel12A* in the analyzed conditions. The expression level of *cel12A* in non-buffered BM media supplemented with 1% rice straw as a sole carbon source was arbitrarily assigned as the reference sample with a $2^{-\Delta\Delta Ct}$ value of 1.0.

In silico analysis of putative transcription factors (TF) and TF binding sites in the promoter of cel12A

The recent availability of the genome sequence of *S. chartarum* IBT 40288 (https://fungi.ensembl.org/Stachybotrys_chartarum_ibt_40288_gca_000732765/Info/Index) was used to obtain the 5′-upstream region (800 bp) of

Table 1 Primers used in this study

Name of the primer	Sequence (5′-3′)	Expected size (pb)
Cel12a_Fwd	TCGTTTCTTGGATTGGGT	116
Cel12a_rev	GGTAGAGTGATTAGGGTT	116
Cel12a_taqman	CGGATGTTGTTGCCCTGGT	TaqMan probe
rRNA18S_Fwd	TGACTCAACACGGGGAAA	116
rRNA18S_rev	CACCCCACCAACTAAGAA	116
rRNA18S_taqman	ATGCACCACCACCCACAAA	TaqMan probe

cel12A and also to search (BLASTP; Altschul et al. 1997) for potential homologs to known fungal transcription factors that could be involved in the regulation of *cel12A*.

Results and discussion

Gene structure of cel12A and promoter analysis

The *cel12A* gene of *S. atra* BP-A (GenBank accession no. AM180511) contains an 848 bp open reading frame (ORF) interrupted by two introns of 78 and 53 bp in length (Picart et al. 2012). Its 5′ untranslated region (*cel12A*$_p$: 800 bp) was in silico analyzed (SnapGene Viewer) for the presence of DNA sequence motifs known to be recognized by relatively well-conserved Zn2Cys6 (i.e., CLR-1/ClrA, CLR-2/ClrB, Ace2/AceB, and XlnR/Xyr1/Xlr-1) and Cys2His2 (CreA/CRE1 and PacC/PAC1) transcription regulators of cellulase genes. As shown in Fig. 1, one (5′-YAGAAT-3′; at position − 345 from the ATG-initiation codon) and three (5′-GGCWWW-3′; positions − 240, − 457, − 689) predicted DNA binding sequences for CLR-2 and XlnR, respectively (Benocci et al. 2017), were found in *cel12A*$_p$, suggesting an in vivo positive role for these activators in the expression of *cel12A*. Neither sites for CLR-1 nor for Ace2 were identified. The transcription activators CLR-2 and its ortholog ClrB are essential for cellulase gene expression in *N. crassa* and *A. nidulans*, respectively (Coradetti et al. 2012). Using the amino acid sequence of CLR-2/NCU08042 of *N. crassa* against the hypothetical proteome of *S. atra* (BLASTP), we found a highly homologous protein (KFA75600/A0A084RHB5, > 70% identity) to CLR-2. Reciprocal BLASTP analysis confirmed that KFA75600 might be the cellulose regulator CLR-2 of *S. atra*. In addition, the Zn(II)2Cys6 DNA-binding domains of both CLR-2 orthologs are 100% identical suggesting that their DNA targets would also be conserved. On the other hand, the transcription activator XlnR regulates the expression of cellulase and hemicellulase genes in *A. niger* (van Peij et al. 1998; Stricker et al. 2008), whereas its ortholog (XLR-1) in *N. crassa* has a different role and predominantly regulates the expression of xylanolytic genes (Sun et al. 2012). BLASTP analysis using the amino acid sequence of the xylanolytic activator (XLR-1/NCU06971) of *N. crassa* revealed that *S. atra* also have an ortholog protein to XLR-1 (i.e., KFA73621/A0A084RBN6). The identity (100%) in the Zn(II)2Cys6 regions of both XLR-1 orthologs suggests that they recognized identical DNA targets.

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                                    -800 ACCCGGATCGGCCCGAACCAGACTTCAGTACCCAA
-765 GGCCCCATAACCGAAACCTTCATTATAAGCGCGCTAATCAAGCGAGTGATGCTGACCCCTTGCT
-701 TCTATGCATTGCCATTTCAGTGCTATTGCGTACTCAACCAAAGTTTGGTTCGCTCCGATAGATA
-637 GATTAATGCTGAAGCAAAGGCCCGAAAACCCTTGTGCATGGCCTGAGGTCCACTTCCTAATGGG
-573 GATGCTGCTTAGACTTGCGATTCGTTGTGGCTGTAGATTGGTCCCCCCCGGGCCATGCAGTTAA
-509 TTTATAAATTGATGAGCTATTAAGGTACAGCAAGCTCATAGAAACACATTGCCAAGGAAACACC
-445 ATGCAACAGGTGGAGAATCTTGGCTGCGATTAGCAAGTTGAGGGCAACCGCAGCTAGCCAAATA
-381 ACCAACCACCAAGTGATGGCCTTCAGTCGACCAAGACAGAATGCTCCGATGGAGTACTATAGTC
-317 ATTCATATGACACACCCAGTGTCATGCACCATTGTTTGTAACCGCTTACCTTTGGTCATGGGCC
-253 CCGAGTGCTAAGCCAACGAGATGTTGCATTCCCCTGGATGAACCCCTTTGGAAACGGACATAGT
-189 CAACCGAACATCTGGGGTCTGGGGTCAAGGCTCCGTTCAAACACAAGATGGCTTCGAGCTCTGA
-125 AGCTGCTATAAGTACATTGGGATCTATCTGGCGATCAGACGAACAGAGCCAGATGAAGCAACTT
-61  GCCAGAACAGTATCTTCAATTGATCATCGTCAACAAGGTTCAAGCCTGCTAGCTGTCAAACATG
                                                                    +1
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Fig. 1 DNA sequence of the *cel12A* gene promoter of *S. atra* BP-A. Putative TATA box and the translational initiation codon are shown in bold. The four DNA sequences (two 5′-CTGGGG-3′, one 5′-GTGGAG-3′ and one 5′-CCGGGG-3′) conforming the consensus binding site for CreA (5′-SYGGRG-3′) are shown in bold and underlined whereas those for PacC (two 5′-GCCAAG-3′) are shown double underlined. The putative DNA binding site (5′-CAGAAT-3′) for CLR-2 is underlined, whereas those for XlnR (one 5′-GGCTTA-3′ and two 5′-GGCAAT-3′) are shadowed in gray. Although these sequences are located on both strands, only the top strand is indicated

Four DNA sequences matching the hexanucleotide 5′-SYGGRG-3′ described as the binding site for the wide-domain glucose repressor CreA (Kulmburg et al. 1993; Cubero and Scazzocchio 1994) were also found in *cel12A*$_p$ (Fig. 1). Two of these sequences are located at positions − 523 and − 436 from the ATG codon, whereas the other two are adjacent and directly oriented sequences separated by one base pair (5′-CTGGGGnCTGGGG-3′; positions − 178 and − 171 from the ATG), strongly suggesting that the expression of *cel12A* is subjected to CCR directly mediated by CreA. It is in this regard noteworthy that a physiological role for CreA sites conforming the sequence 5′-CTGGGG-3′ has been shown in both the intergenic region between *prnD* and *prnB* (Cubero and Scazzocchio 1994) and the promoter region of the xylanase gene *xlnA* (Orejas et al. 1999) of *A. nidulans*. BLASTP and reciprocal BLASTP analyses revealed that *S. atra* also has one hypothetical protein (KFA75113/A0A084RFX8) ortholog to the CreA repressor (AN6195) of *A. nidulans*.

Finally, two copies of the DNA consensus sequence 5′-GCCARG-3′, which is the binding site of the main regulatory protein (PacC) controlling pH-dependent transcriptional regulation in *A. nidulans* (Caddick et al. 1986; Tilburn et al. 1995) and other fungi, were detected in *cel12A*$_p$ at positions − 422 and − 459 from the ATG (Fig. 1). This observation suggests that expression of *cel12A* could be also controlled by the pH/PacC regulatory circuit. Using the BLASTP program, PacC (AN2855) has been also shown to have one putative ortholog in *S. atra* (KFA79600/A0A084RTR5).

In summary, in silico analysis of the 5′ flanking region adjacent to *cel12A* resulted in the identification of motifs and promoter elements that could be involved in its regulation. BLAST searches against the proteome of *S. atra* resulted in the identification of hypothetical regulatory proteins that could bind to those promoter targets to modulate the expression of *cel12A*. Rational engineering of these transcriptional factors could allow to enhance the production of not only Cel12A but also other industrially important PCWDE in *S. atra* or other *Stachybotrys* species (according to the JGI Genome Portal MycoCosm, the genome of *S. elegans MPI-CAGE-CH-0235* has potential to encode 841 carbohydrate acting enzymes, while that of the industrial enzyme producers *A. niger* and *T. reesei* would encode 516 and 407, respectively). A similar strategy has been successfully applied to enhance the production of endogenous and heterologous PCWDEs in *A. nidulans* (Tamayo-Ramos and Orejas 2014). Moreover, as a biocontrol activity has been suggested for some *Stachybotrys* species, and expression of genes encoding extracellular enzymes associated with mycoparasitism (proteases, β-glucanases, chitinases, and other fungal cell wall-degrading enzymes) could be also controlled by the carbon source and ambient pH, the identification of the potential regulatory proteins KFA79600/PacC and KFA75113/CreA of *Stachybotrys* could provide a better knowledge of the molecular mechanism involved in its mycoparasitism and thus help to improve its biocontrol efficiency against fungal pathogens.

Influence of carbon sources and ambient pH on lichenase production

The potential regulatory elements found in the promoter of *cel12A* as well as the activity of its gene product suggested that expression of *cel12A* could be at least regulated by two abiotic factors: carbon source (induced by cellulosic/hemicellulosic substrates and repressed by glucose) and ambient pH. Regarding to the inducing carbon source, current research into PCWDEs focuses on their uses in the bioconversion of agricultural wastes, as well as the environmental benefits that could have the use of these substrates. It has been reported that rice straw (one of the abundant lignocellulosic waste materials in the world; Karimi et al. 2006) greatly induced the production of cellulases in *S. atra BP-A* (Picart et al. 2008) and β-glucanases in *Aspergillus terreus* ASKU 10 (Prajanban et al. 2008). On the other hand, in the CCR/CreA system of *A. nidulans*, lactose is considered to be a non-repressing carbon source, whereas 0.1% glucose is less repressing than 1% glucose (Bailey and Arst 1975).

Previous studies in our group of research (unpublished results) showed the presence of at least three protein bands in SDS-PAGE which zymography revealed to have lichenase activity under rice straw conditions. Bearing this study in mind along with the abovementioned considerations, to assess whether lichenase biosynthesis in *S. atra* is indeed regulated by the carbon source, extracellular lichenase activity was firstly compared in transfer experiments from 1% glucose to potential inducing (1% rice straw), non-repressing (1% lactose), and two different levels of repressing conditions (1% glucose and 0.1% glucose). As seen in Fig. 2, the complex source of carbon rice straw (predominantly contains cellulose (30–47%), hemicellulose (19–27%), and lignin; Karimi and Taherzadeh 2016; Jin and Chen 2007; Horikawa et al. 2011) was the most effective inducer among the carbon sources tested. In the sole presence of this substrate, extracellular lichenase activity was 6-fold greater than that obtained in 0.1% glucose (0.36 vs 0.06 U/mL), while it was not detected in 1% lactose or 1% glucose. These results suggest that in *S. atra*, lichenase activity is produced at a basal level which is induced by rice straw and repressed by glucose and lactose.

To assess whether glucose also represses lichenase induction, extracellular lichenase activity was studied in transfer experiments to rice straw media with and without glucose. Figure 2 shows that lichenase activity was abolished when 1% glucose was combined with the inducer indicating that the induction of lichenase in *S. atra* is under CCR. As expected, lichenase activity was about 50% lower in media co-supplemented with rice straw and 0.1% glucose (0.19 U/

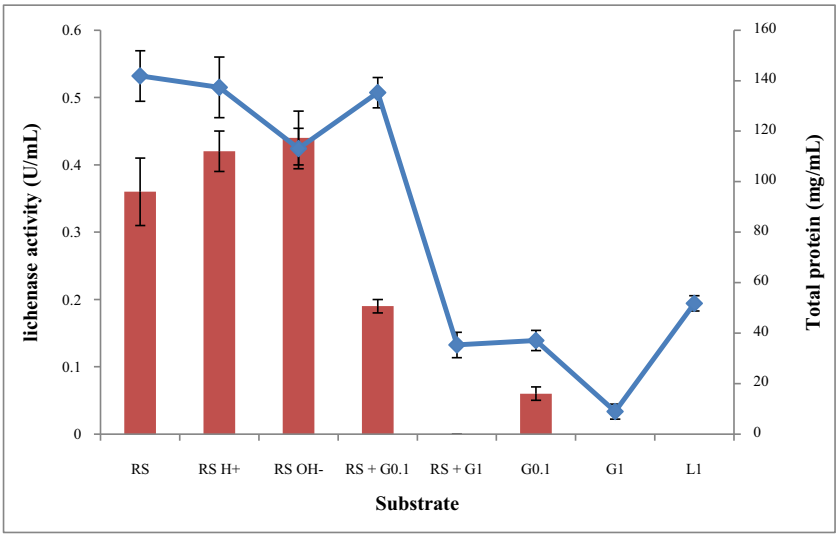

Fig. 2 Influence of the carbon source and ambient pH on *S. atra* BP-A lichenase production. Rice straw at 1% and rice straw at 1% buffered at two different initial pH conditions (acidic 5.2, and alkaline 7.9), glucose at 1% and 0.1%, and lactose at 1%, used individually and combined with RS at 1%. Bars represent the lichenase activity (mean ± standard deviation), whereas the line shows the total extracellular protein concentration (mean ± standard deviation) (RS, raw straw; RS H+, raw straw adjusted at acidic pH 5.2; RS OH-, raw straw adjusted at alkaline pH 7.9; G1, 1% glucose; G0.1, 0.1% glucose; and L1, 1% lactose)

mL) than in the sole presence of rice straw, indicating that induction is partially repressed under low concentration of the repressor (glucose). Not surprisingly, the amount of secreted proteins varied depending on the nature of the carbon source (Fig. 2). In the presence of complex substrates such as rice straw, more enzymes (lichenase and other PCWDEs) should be induced than in its absence or in the presence of repressing conditions (1% glucose) where biosynthesis of most of these enzymes is negatively affected.

It can therefore be concluded that lichenase activity (including Cel12A) in *S. atra* is induced by rice straw and this induction repressed by glucose. In a previous study, we have also found that *S. atra* BP-A secreted high cellulase activity when grown on rice straw, and this is not produced in the presence of glucose (Picart et al. 2008), suggesting that lichenase and cellulase genes might be co-regulated, and their promoters would share common *cis*-acting elements. The production profile of lichenase activity in *S. atra* is also similar to that of the *Stachybotrys microspore* endoglucanase, which is highly produced on cellulosic substrates such as wheat bran, and repressed on lactose and glucose (Ben Hmad et al. 2014), and to that of *P. occitanis* lichenase, where its production is greatly induced by cellulose, barley flour, barley bran, and oat flour (Chaari et al. 2014).

Finally, to investigate the effect of ambient pH on lichenase production, lichenase activity was studied in transfer experiments to inducing media (1% rice straw) buffered at acidic (pH 5.2) or alkaline (pH 7.9) pH values. As seen in Fig. 2, both pH conditions gave similar yields of extracellular lichenase activity (about 0.4 U/mL), suggesting the lack of a role for pH and PacC in the synthesis of lichenase. Similar yields were also reached in the presence of phosphate (0.36 U/mL) indicating the lack of a role for this supplement. Interestingly, most of cellulolytic fungi show an acidic pH optimum for PCWDEs secretion (Chaari et al. 2014; Elgharbi et al. 2013; Grishutin et al. 2006; Murray et al. 2001). Instead, *S. atra* BP-A produced Cel12A at both acidic and alkaline pH, a behavior that seems not to be very often among fungi.

Analysis of cel12A expression in response to carbon source and ambient pH

To investigate whether the results obtained at the lichenase activity level correlated with differences in the expression of *cel12A*, its steady-state mRNA levels were quantified (by RT-qPCR) in mycelia obtained from the same cultures used to measure lichenase activity. The housekeeping gene *18S rRNA* was firstly validated to normalize *cel12A* expression data. As shown in Table 2, *18S rRNA* displayed similar C_t values across all experimental samples, indicating that its expression is stable in these conditions and it can therefore be considered a suitable reference gene. We have validated the *18S rRNA* as a reference gene for RT-qPCR analysis in *S. atra*, and we expect that it could potentially be used to normalize other results of gene expression in this fungus.

In contrast to the stable expression of *18S rRNA*, the C_t values of *cel12A* varied widely among the different growth conditions analyzed (Table 2), indicating that its expression is regulated. We arbitrary assigned as a reference sample the C_t value derived from the cultures of *S. atra* BP-A in 1% rice straw as a sole carbon source (considered to be an inducing condition for lichenase activity, see above). To assess whether rice straw also induced gene expression of lichenase genes, Cel12A transcripts from mycelia grown either in 1% rice straw, 1% lactose, or 0.1% glucose were quantified. Table 2 shows that the relative mRNA levels of *cel12A* are elevated in rice straw in comparison to those in the sole presence of 1% lactose (about 11-fold) or 0.1% glucose (about 58-fold), indicating that transcription of *cel12A* is indeed activated by rice

Table 2 Relative expression of *cel12A* and *18S rRNA* in *S. atra* BP-A

Sample	Mean $C_{tCel12A}$	Mean $C_{tRNA18S}$	ΔCt ($C_{tCel12A}$ -$C_{t18S\ rRNA}$)	$\Delta\Delta C_t$	$2^{-\Delta\Delta Ct}$ (-fold)
1% Rice straw	29.35 ± 0.305	15.537 ± 0.015	14.108 ± 0.124	0.0 ± 0.124	1.0 ± 0.124
1% Rice straw pH 5.2	28.413 ± 0.244	15.85 ± 0.111	12.563 ± 0.268	− 1.545 ± 0.268	2.918 ± 0.596
1% Rice straw pH 7.9	28.576 ± 0.069	16.163 ± 0.051	12.412 ± 0.086	− 1.696 ± 0.087	3.24 ± 0.195
1% R straw + 0.1% glucose	33.71 ± 0.325	15.503 ± 0.075	18.207 ± 0.334	4.099 ± 0.334	−17.136 ± 3.546
1% R straw + 1% glucose	**38.98** ± 0.352	14.94 ± 0.026			
0.1% Glucose	35.123 ± 0.201	15.16 ± 0.026	19.963 ± 0.203	5.855 ± 0.203	−57.88 ± 8.745
1% Glucose	**38.973** ± 0.140	15.547 ± 0.032			
1% Lactose	32.637 ± 0.238	15.10 ± 0.040	17.537 ± 0.33	3.429 ± 0.33	−10.77 ± 2.769
Distilled water	**39.19** ± 0.26	38.647 ± 0.608			

Values are expressed as the means with their standard deviation. The expression level of *cel12A* in 1% rice straw was arbitrarily assigned as the reference sample with a $2^{-\Delta\Delta Ct}$ value of 1.0. $C_{tCel12A}$ values in bold correspond to values found for water and thus represent no expression of *cel12A*

straw. Likewise, *cel12A* mRNAs accumulated upon transfer to 1% rice straw but not (the C_t values are almost identical to those of the water control, about 39) in mycelia transferred to 1% rice straw + 1% glucose, or to 1% glucose alone (the pre-growth condition), clearly indicating that 1% glucose totally represses the expression of *cel12A* (with or without the inducer). Repression of the induction of *cel12A* is less pronounced at low glucose concentrations, as reflected by the amount of *cel12A* transcripts that was reduced 17-fold when the mycelia were grown in rice straw + 0.1% glucose relative to growths in 1% rice straw (Table 2). Great repression by 0.1% glucose of *cel12A* was observed in the absence of the inducer (about 58-fold less mRNA in 0.1% glucose than in 1% rice straw, and a high C_t value > 35). Qualitatively, these results are in good agreement with those of lichenase activity (Fig. 2). The exception is the response of *S. atra* to 1% lactose. While this carbon source allows a certain expression of *cel12A*, extracellular lichenase activity was undetectable. One possible explanation to this result is a possible degradation of Cel12A by protease(s) produced in the presence of lactose. Protease production in fungi under specific growth conditions has already been reported (de Souza et al. 2015). Similar to *cel12A*, moderate expression of some *T. reesei* cellulolytic genes in the presence of lactose has also been reported (Amore et al. 2013 and references therein).

Transcription of *cel12A* was also analyzed in mycelia grown in 1% rice straw under acidic (pH 5.2) and alkaline (pH 7.9) conditions. In agreement to the lichenase activity data (Fig. 2), transcript levels of *cel12A* were very similar under both pH conditions (Table 2), indicating that transcription of *cel12A* is independent of the pH/PacC regulatory circuit as well as the lack of a physiological role for the three consensus PacC target sites in $cel12A_p$. It is interesting that acid or alkaline pH conditions are not affecting—as it seems in Fig. 2—the total extracellular lichenase activity, whereas the mRNA of *cel12A* (Table 2) accumulated at greater levels in buffered media than under control. It can be speculated that other lichenase activities could be masking the expression profile of *cel12A*.

Taken together these results demonstrate that both rice straw induction and glucose repression (CCR) of lichenase production in *S. atra* take place at the level of transcription of at least *cel12A*. The presence of potential target sequences for the cellulase/hemicellulase transcription activators CLR-2 and XlnR in $cel12A_p$ suggests a positive role for their orthologs (KFA75600 and KFA73621, respectively) activating *cel12A* transcription in the presence of raw straw. Likewise, the presence of four CreA consensus binding sites in $cel12A_p$ would suggest direct repression of *cel12A* by CreA/KFA75113 in the presence of glucose with and without rice straw. With regard to the molecular mechanism by which CreA/KFA75113 would repress *cel12A* transcription, the fact that the potential CLR-2, XlnR, and CreA targets do not overlap would rule out direct competition between CreA and the potential transcription activators.

Conclusions

In this study, we report on the expression of a gene encoding an extracellular lichenase activity in the genus *Stachybotrys*. From 2015, the genome sequence of *S. chartarum* (AKA *S. atra*) is publicly available (Betancourt et al. 2015). Its in silico analyses resulted in the identification of transcriptional factors orthologs to PacC, CreA, CLR-2, and XlnR, which regulate the expression of PCWDEs in several filamentous fungi. Further manipulation of these control systems (e.g., rational engineering of CreA to remove CCR) may significantly improve yields of Cel12A and another PCWDEs in *S. atra*. Taking into account the key role of lichenases in biotechnological applications (e.g., brewing, animal foodstuffs) as well as the fact that these enzymes are an essential component of the enzyme portfolio for degrading the lignocellulose resources to produce biofuel (Dashtban et al. 2010),

it is evident that new knowledge about the biochemical properties of these enzymes and the regulatory mechanisms involved in their production in various microorganisms is still necessary. Consequently, we believe that the present work will set the basis for further research on the genus *Stachybotrys* and will encourage other scientists to study the transcriptional regulation and recombinant production of new PCWDEs produced by these important cellulolytic fungi. In addition, open-field burning of rice residues has harmful environmental implications, and thus the use of this substrate to generate value-added products such as ethanol or enzymes could help to alleviate problems related to their disposal. In this regard, since promoters are key tools in biotechnology to ensure that gene expression is effective, rice straw responding promoters (such as $cel12_p$) for the overexpression of transgenes could well be an interesting alternative to valorize this agriculture by-product.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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