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RESEARCH ARTICLE

A novel luciferase-based reporter tool to monitor the dynamics of carbon catabolite repression in filamentous fungi

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Abstract

Filamentous fungi with their diverse inventory of carbohydrate-active enzymes promise a holistic usage of lignocellulosic residues. A major challenge for application is the inherent repression of enzyme production by carbon catabolite repression (CCR). In the presence of preferred carbon sources, the transcription factor CreA/CRE-1 binds to specific but conserved motifs in promoters of genes involved in sugar metabolism, but the status of CCR is notoriously difficult to quantify. To allow for a real-time evaluation of CreA/CRE-1-mediated CCR at the transcriptional level, we developed a luciferase-based construct, representing a dynamic, highly responsive reporter system that is inhibited by monosaccharides in a quantitative fashion. Using this tool, CreA/CRE-1-dependent CCR triggered by several monosaccharides could be measured in *Neurospora crassa*, *Aspergillus niger* and *Aspergillus nidulans* over the course of hours, demonstrating distinct and dynamic regulatory processes. Furthermore, we used the reporter to visualize the direct impacts of multiple CreA truncations on CCR induction. Our reporter thus offers a widely applicable quantitative approach to evaluate CreA/CRE-1-mediated CCR across diverse fungal species and will help to elucidate the multifaceted effects of CCR on fungal physiology for both basic research and industrial strain engineering endeavours.

INTRODUCTION

Filamentous fungi play a major role in biotechnology, as they harbour a complex metabolism and have the ability to degrade vast amounts of different plant biomass residues and produce various metabolites. Hence, these microbes are heavily used in industry and science (Aristidou & Penttilä, 2000; Sauer et al., 2008; Schäfer et al., 2019; Schmitz et al., 2019). On the other hand, pathogenic ascomycetes have a huge effect on agricultural production and human health (Baxi et al., 2016; Lucca & Anthony, 2007; Timberlake & Marshall, 1989). The production of industrially important plant cell wall hydrolases, secondary metabolites and toxins is tightly regulated by several inducing and repressing pathways. One highly conserved control mechanism is carbon catabolite repression (CCR) (Adnan et al., 2017; Gancedo, 1998; Matar et al., 2017; Revilla et al., 1984; Ries et al., 2021; Shinmyo et al., 1978; Tamayo et al., 2008). Upon sensing readily available carbon sources like glucose or other monosaccharides, filamentous fungi stop redirecting their metabolism towards obtaining difficult-to-exploit substrates to avoid wasting energy (Adnan et al., 2017; de Vries

et al., 1999; Gancedo, 1998; Kowalczyk et al., 2014; Peng et al., 2021; Ruijter & Visser, 1997). Furthermore, various processes like regulation of growth, hyphal morphology, sporulation, pathogenicity and symbiosis are also tightly regulated by CCR, drawing the importance of understanding its underlying regulatory mechanisms (Fellbaum et al., 2012; Franzino et al., 2022; Matar et al., 2017; Nakari-Setälä et al., 2009; Ries et al., 2021).

The main regulator of CCR is the Cys_2His_2 zinc finger transcription factor CreA/CRE-1 (Cre), which is highly conserved throughout fungi (Dowzer & Kelly, 1989, 1991; Nehlin & Ronne, 1990). Upon repressing conditions, when favourable carbon sources are present, Cre is phosphorylated in its alanine-rich domain and in a region that is highly conserved among *Aspergillus niger*, *Aspergillus nidulans* and *Trichoderma reesei*, and transported to the nucleus (Brown et al., 2013; de Assis et al., 2021). In the nucleus, Cre binds via its zinc finger domain to specific binding sites in the promoter regions of various genes, leading to their repression by displacing activators from their target regions or by indirectly hindering their action. Thereby, Cre directly exerts repression of genes responsible for sugar catabolism, sugar transport and plant cell wall degradation or indirectly by repressing their activators (Wu et al., 2020). Throughout the phylum of Ascomycota, 5′-SYGGRG-3′ repeated motifs were found to be the target sequences for Cre (Ruijter & Visser, 1997; Sun & Glass, 2011). In *Neurospora crassa*, up to 318 promoter regions were found to contain CRE-1-binding motifs and 271 of the associated genes were upregulated upon CRE-1 deletion (Sun & Glass, 2011; Wu et al., 2020). These genes included cellulolytic and hemicellulolytic genes as well as their corresponding transcriptional activators (de Vries et al., 1999; Ruijter et al., 1997; Strauss et al., 1995; Sun et al., 2012; Sun & Glass, 2011). During derepression conditions in *A. nidulans*, CreA is bound to the protein kinases GskA and CkiA and translocated to the cytoplasm (de Assis et al., 2018, 2021). Furthermore, degradation of CreA is accelerated by CreD-HulA (arrestin motif protein–ubiquitin ligase) complex-dependent ubiquitination (Alam et al., 2016; Lockington & Kelly, 2001).

To reduce the negative effects of CCR during industrial enzyme production, Cre is typically knocked-out, but due to the central importance of Cre, this creates various other phenotypes leading to a loss of fitness (Rassinger et al., 2018; Robl et al., 2018; Shroff et al., 1997). To approach these downside effects, molecular engineering has been used to generate derepressed fungal strains, for example, by introducing Cre mutations (de Assis et al., 2021; Eveleigh & Montenecourt, 1979; Mello-de-Sousa et al., 2014; Rassinger et al., 2018; Ries et al., 2016; Shroff et al., 1996; Zhang et al., 2016). Moreover, the industrial cellulase producer strain *T. reesei* RUT-C30, which was created by rounds of classical mutagenesis, was found to carry a nonsense mutation at codon 97 that generated a truncated version of Cre1 which exerts activator characteristics (Rassinger et al., 2018). Based on these findings, different truncations were generated in *A. nidulans* harbouring various domains of the repressor to gain insight into the function of these domains (Ries et al., 2016).

Due to the significance of CCR in fungal metabolism, different methods to measure CCR have been established. Phenotypic assays containing the glucose analogue 2-deoxyglucose (2-DG) and allyl alcohol are commonly used to visualize CCR effects on biomass production in mutant strains (Hicks et al., 2001; Shroff et al., 1997). To test CCR repressiveness of different fungal strains, 2-DG is added to minimal medium plates or liquid cultures along with a carbon source which is less favoured compared to glucose. 2-DG cannot be catabolized as carbon source, but triggers CCR, leading to an inhibition of catabolism and therefore an inability to use the less favoured carbon source in the medium despite its presence. Allyl alcohol, on the other hand, is metabolized by alcohol oxidases in derepressed strains to the toxic degradation product acrolein, which leads to growth inhibition. Despite their usefulness, precise quantification of CCR cannot be carried out with these assays, and due to end-point measurements, a real-time observation of CCR effects is not possible. A more direct but elaborate way to assess CCR is to tag the main CCR regulator Cre with fluorescent proteins and visualize the CCR-dependent translocation of Cre to the nucleus. However, this can only give a broad picture of CCR effects, and it was shown that the localization of Cre in the nucleus is not a key regulatory mechanism of CCR (Roy et al., 2008). The stated methods above are applied to screen for strains with derepressed CCR, for example, to find strains suitable for industrial applications to produce enzymes and metabolites in complex media-containing glucose or other easily metabolizable carbon sources (Kowalczyk et al., 2014). Overall, currently available methods lack the precision to accurately quantify CCR effects in real-time. They are intricate and not easily transferable to different conditions and fungal strains due to their inherent limitations. In this work, we achieved to develop a novel reporter tool fulfilling all those needs applicable to mycelia of submerged cultures and from growth on solid substrate.

EXPERIMENTAL PROCEDURES

Filamentous fungal strains and culture conditions

The *A. niger* and *A. nidulans* strains depicted in the Table S2 were cultivated in *Aspergillus* minimal medium (aMM) with aMM salts and 2% glucose as carbon

source or in *Aspergillus* complete medium (aCM) which is based on aMM with addition of 2% glucose, 0.5% yeast extract and 0.1% casamino acids (Arentshorst et al., 2012). Conidia were generated on aCM combined with potato extract glucose agar in the concentration of 39 g/L (Roth). They were then harvested with 0.89% NaCl, and 0.05% of Tween 80 solution after 10 days of cultivation at 30°C for *A. niger* and 25°C for *A. nidulans* under constant light. One mM uridine, 21 μM nicotinamide and 29.6 μM pyroxidine were added to the medium when needed.

Neurospora crassa strains (Table S2) were cultivated on *Neurospora* MM (nMM) containing 1× Vogels salts for MM and 2% sucrose as carbon source under constant light and 25°C for 7 days to generate conidia (Vogel, 1956).

Cloning of the CCR reporter cassettes

For the general cloning procedure, the protocols of Sambrook and Russell (2001) were followed to build the reporter cassette containing a firefly luciferase coding sequence under the control of a CCR inhibitable promoter. The *N. crassa* codon optimized *Photinus pyralis* luciferase coding sequence was amplified from the plasmid pXM1.1 (van Gooch et al., 2008) while removing the peroxisomal targeting sequence as described by Leskinen et al. (2003). To ensure a quick responsiveness to CCR, a C-terminal PEST-signal peptide was fused to the luciferase for a rapid protein turnover (Cesbron et al., 2013).

To build an inducible reporter system, the promotor was engineered based on the Tet-on system established by Meyer et al. (2011) for filamentous fungi. It comprises seven repeats of the tetracycline resistance operon (*tetO7*) coupled with the downstream located glyceraldehyde-3-phosphate dehydrogenase minimal promoter (P_{min}*gpdA*) which is regulated by the tetracycline-inducible transactivator rtTA2S-M2 (Meyer et al., 2011). Furthermore, two different Cre-1-binding motifs found in promoter regions of the *N. crassa* cellulolytic regulon were introduced between the *tetO7* site and the minimal promoter P_{min}*gpdA* to generate Cre-mediated responsiveness (Sun & Glass, 2011). The 5′-GTGGGG-3′-binding motif was used in 2, 4 or 8 identical repeats or 2, 4 or 8 alternating repeats in combination with 2, 4 or 8 5′-GCGGGG-3′ motifs spaced by 5′-AA-3′. In addition, a construct containing 8 5′-GCGGGG-3′ motifs spaced by 5′-AA-3′ was created. To generate Cre-independent control constructs, spacer sequences of the same length as the 4× and 8× Cre-binding site fragments were inserted into the same construct in place of the Cre-binding sites (5′-ATCTATTACTTCTGTACTACCATTCCATAC-3′ and 5′-ATCTATATACTACAAAATTACTTTACTCCCATCTCAATTACTTCTGTACTACCATTCCATACAA-3′) (Table S1). These Cre-1-binding sequences were cloned by annealing DNA oligonucleotides with overhangs suitable for integration into the synthetic promoter (Eurofins Genomics). In combination with the rtTA2S-M2 transactivator cassette, the reporter was inserted into multicassettes backbones for genome integration and auxotrophy complementation using BsmBI-based Golden Gate cloning (Lee et al., 2015).

Cloning of the *A. niger creA* truncation constructs

Deletion constructs for building part plasmids of the *creA* gene in *A. niger* were constructed by using Gibson assembly. This involved cloning the *creA* 5′-flank consisting of approximately 900 base pairs upstream of *creA* (bp) along with the remaining truncated coding sequence, and a 3′-flank starting with the stop-codon (968 bp) using the gDNA of *A. niger NRRL3* as template. These sequences were integrated into the plasmid pYTK095 obtained from AddGene including NotI restriction site flanks enabling the generation of linear repair templates prior to *A. niger* transformation (Lee et al., 2015). To increase the rate of homologous recombination, a self-replicating CRISRPR/Cas9 (clustered regularly interspaced short palindromic repeats; CRISPR-associated protein 9) plasmid pKS017, constructed as described in Knesebeck et al. and containing the single-guide RNA 5′-GACATCATGTCTAAACCGGA-3′, was co-transformed promoting a double-strand break in the *creA* genomic region (Table S1; Knesebeck et al., 2023; Leynaud-Kieffer et al., 2019). Using this approach, truncation strains were established, each carrying a shortened version of CreA encompassing the amino acids (aas) 1–115, 1–162, 1–281 and 1–345. These truncations were designed based on homologous truncations created in *A. nidulans* by Ries et al. (2016) removing functional domains of the repressor (Ries et al., 2016). Furthermore, a deletion of the aa 281–345 was created following this protocol.

Genetic manipulation of filamentous fungi

Aspergillus niger and *A. nidulans* protoplasts were transformed according to the established protocol of Niu et al. (2016). To generate CCR reporter strains of *A. niger*, the reporter construct was introduced in a *nicB*- and *kusA*-deficient strains with CCR reporter multicassettes plasmids integrating into the *NicB* locus and therefore complementing the nicotinamide auxotrophy (Meyer et al., 2007). *Aspergillus nidulans* was transformed by ectopic integration of a *pyrG*-complementing multicassette also containing the CCR reporter. *CreA* truncations were established in a *pyrG*- and *kusA*-deficient *A. niger* strain by CRISPR/Cas9-mediated

sequence deletions with self-replicating plasmids containing a *pyrG* cassette for selection. For genomic validation of truncated strains via PCR of the genomic target loci, genomic DNA was extracted using the established protocol (Figure S5) (Knesebeck et al., 2023). After strain validation, counter selection was performed to eliminate the CRISPR/Cas9 plasmid using 1 mM uridine and 0.75 mg/mL 5-fluorooratic acid-supplemented aMM 1.5% agar plates. *N. crassa* was transformed by electroporation complementing the histidine auxotrophy established by Chakraborty and Kapoor (1990) and homokaryons were obtained by three rounds of singulation for *Aspergillus* strains or enrichment of microconidia for *N. crassa* (Ebbole & Sachs, 1990; Meyer et al., 2010).

CCR-based luciferase assays

In white 96-well microtiter plates with transparent bottoms and Breathe-Easy® sealing membranes, 10^6 spores/mL were cultivated per well in 200 μL of aMM + 0.5% glucose or nMM + 0.5% sucrose and the corresponding supplements. Cultivation was carried out at 30°C and 130 revolutions per minute (rpm) for 21 h for *A. niger* and *A. nidulans* and 5 h for *N. crassa*. The mycelium adheres to the bottom of the wells, which facilitates the removal of the culture medium. After removing the sealing membrane and the supernatant, the mycelium was washed four times with 200 μL of the corresponding MM without carbon source for 45 min to induce starvation.

After addition of 200 μL MM, supplemented with 1 mM D-luciferin potassium salt (Cayman chemicals) and 12 μg/mL doxycycline, the plate was inserted into a Tecan SPARK plate reader. While keeping the plate sealed with a membrane, the plate was shaken at 30°C at 130 rpm. The OD_{600} and luciferase values were measured every 8.5 min for each well. After 1.5 h of starvation for the *Aspergilli* strains and 45 min for *N. crassa*, the luciferase signal reached the exponential phase. At this time point, the sealing membrane was removed and different monosaccharides were added to individual wells to assess their influence on Cre-mediated CCR. After adding a new sealing membrane, the measurement was resumed.

2-Deoxy-2-glucose (2-DG) assay

Plates of 20 mL solid aMM (1.5% agar) with 1% xylose with and without 20 mM 2-DG were inoculated with 2 μL of 10^8 spores/mL in the centre and incubated at 30°C for 7 days (de Assis et al., 2018). Growth diameter was measured for all colonies using ImageJ (Schindelin et al., 2012). The difference in strain diameter observed on plates supplemented with both xylose and 2-DG, in contrast to strains grown solely on xylose plates, was utilized to assess the effects of CCR on pentose catabolism.

Aspergillus niger macroscopic luciferase assays

A piece of nylon membrane close to the size of the petri dish was laid on top of solidified aMM with 2% glucose. Afterwards, 2 μL of 10^8 spores/mL were used to inoculate the plate in the middle and incubated at 30°C for 7 days. The nylon membrane was then peeled off with the biomass on top and washed in a petri dish containing liquid aMM without carbon source but containing all the needed supplements for 15 min. After four rounds of washing, the nylon membrane was incubated in aMM containing 1 mM uridine, 3 mM luciferin and 12 μg/mL doxycycline for 2.5 h. The colonies' luminescence emission was measured at 560 nm wavelength using the Fusion Solo S, Vilber Lourmat gel documentation system with an exposure time of 5 min. For CCR induction, the nylon membrane was transferred into a 50% glucose solution containing 1 mM uridine, 3 mM luciferin and 12 μg/mL doxycycline. After 1.5 h, another picture was taken to assess the responsiveness of the reporter strain to CCR.

Neurospora crassa macroscopic luciferase assays

Neurospora crassa spores (10^6 spores/mL) were cultivated in a 24-deep well-plate containing 3 mL of nMM, 2% sucrose at 30°C and 250 rpm for 24 h. The mycelium was removed with tweezers and washed three times over a time of 45 min by transferring the mycelium into fresh nMM without carbon source. The biomass was then starved for 3 h in nMM without carbon source, supplemented with 1 mM D-luciferin potassium salt (Cayman chemicals) and 10 μg/mL doxycycline at 30°C and 250 rpm. To visualize the luciferase light output, the wavelength at 560 nm was documented using the Fusion Solo S, Vilber Lourmat gel documentation system, for an exposure of 5 min. CCR was induced by addition of glucose to the medium in the deep-well plate to reach a concentration of 5%, followed by a second visualization after 3.5 h of incubation at 30°C and 250 rpm.

Statistical analyses

Data shown in graphs represent the mean of at least three biological replicates for each condition ($n \geq 3$) and error bars correspond to the standard deviation. For data comparing two groups, the Shapiro–Wilk test

for normality was carried out. In cases where one of the treatments (or both) was not normally distributed, a Mann–Whitney *U* test was applied. If the data were normally distributed, a two-tailed Student's *t*-test was applied for the comparison of the conditions. Significance is indicated by asterisks (*$p < 0.05$; **$p < 0.01$).

RESULTS

Engineering and macroscopic evaluation of a 4× Cre-binding site reporter

With the aim to engineer a reporter tool able to quantify Cre-mediated CCR in a real-time approach, a luciferase reporter gene driven by a Cre-regulated synthetic promoter was designed. Luciferase reporters are sensitive and rapid tools to quantify gene expression (Luehrsen et al., 1992). The promoter was based on a doxycycline-inducible Tet-ON promoter (Meyer et al., 2011). To render its responsiveness to CreA/CRE-1 regulation, four repeats of the *N. crassa* CRE-1-binding motif 5′-GTGGGG-3′ were added between the seven repeats of the tetracycline-inducible elements (tetO7) and the glyceraldehyde-3-phosphate dehydrogenase minimal promoter (P_{min}*gpdA*) (Figure 1A,B).

The 4× identical Cre-binding site reporter was initially transformed and tested in *A. niger* (Figure 1A). For this purpose, the reporter construct was compared to a luciferase construct containing an equivalent length spacer sequence without Cre-binding sites (Sun & Glass, 2011). *Aspergillus niger* colonies of these strains grown on solid aMM were compared. To activate the promoter by releasing it from CreA, the strains underwent a 2.5 h period of starvation while supplemented doxycycline enabled luciferase transcription. However, after transfer to 50% glucose strongly attenuated the reporter signal by inhibiting luciferase synthesis. This could not be observed for the control construct with the spacer sequence, demonstrating the CreA-dependence and functionality of the tool.

Comparison of Cre-dependent CCR reporter with different number and types of Cre-binding sites

To optimize the CCR reporter towards tighter control, higher sensitivity and broader dynamic range, a variant of the DNA-binding motif found in *N. crassa* promoter regions of CRE-1-regulated genes (5′-GCGGGG-3′) (Sun & Glass, 2011) was introduced into the synthetic promoter and different repeat numbers were compared. Alternating binding sites were compared to identical sites of 5′-GTGGGG-3′ using 2, 4 or 8 repeats spaced by 5′-AA-3′ and tested in quantitative luciferase reporter assays (Figure 1B). It was visible that 0.5% glucose led to an inhibition of luciferase production after 3.3 h of glucose addition in all tested strains compared to starvation conditions except for the reporter with 2× identical Cre-binding sites (Figure 1C). The 2× Cre-binding site reporter harbouring 5′-GTGGGG-3′ and 5′-GCGGGG-3′ showed a drop in signal at 0.5% glucose to only around 40% (Figure 1F). The luciferase reporters controlled by 4 or 8 identical 5′-GTGGGG-3′ sites showed a similar inhibition pattern as the 2× alternating Cre-binding site reporter and the reporter containing 8× identical 5′-GTGGGG-3′ sites, with a drop in luciferase signal to 40% and 47% in 0.5% glucose, respectively (Figure 1D,E). The reporter showing the highest sensitivity towards glucose was the reporter with 8× alternating Cre-binding sites, which was strongly inhibited by glucose, and for which a signal attenuation of 95% was reached (Figure 1H), closely followed by the 4× alternating Cre-binding site reporter, with about 85% (Figure 1G). For the reporter with 8× alternating Cre-binding sites, higher concentrations of glucose suppressed luciferase emission more strongly than lower concentrations, demonstrating a dynamic and concentration-dependent effect (Figure 1H). After ~8 h, an increase in luminescence is visible for cultures in 0.01% of glucose. The same effect is visible for 0.03% glucose cultures, albeit 2 h delayed. It seems that after this time, enough glucose is consumed to allow for release of the reporter from CCR (Figure S4). Furthermore, a reporter variant with 8× repeats of the 5′-GCGGGG-3′ was tested upon repression in microtiter submerged cultures upon addition with 0.5% glucose but did not show a significantly stronger suppression compared to the 8× alternating binding site promoter after 4.5 h of induction (Figure S1). The repetition and alternation of the tested binding sites thereby created a sensitive CCR reporter tool regulated by CreA in a quantifiable manner, leading to a glucose concentration-dependent inhibition of luminescence. Next, biomass from the 8× Cre alternating binding site reporter strain grown on solid aMM was tested upon luminescence emission under starvation and glucose addition, showing the repression of light emission under CCR conditions and proving the applicability of this construct for macroscopic imaging of CreA-mediated CCR (Figure S2).

Comparison of the Cre-mediated CCR responsiveness of *A. niger* and *N. crassa*

After providing evidence that the 8× alternating Cre-binding site reporter displayed the strongest inhibition by glucose, this reporter was transformed into *N. crassa* and tested in macroscopic luminescence assays with submerged cultures. As observed in *A. niger*, the strain exhibited a reduction in light emission in response to

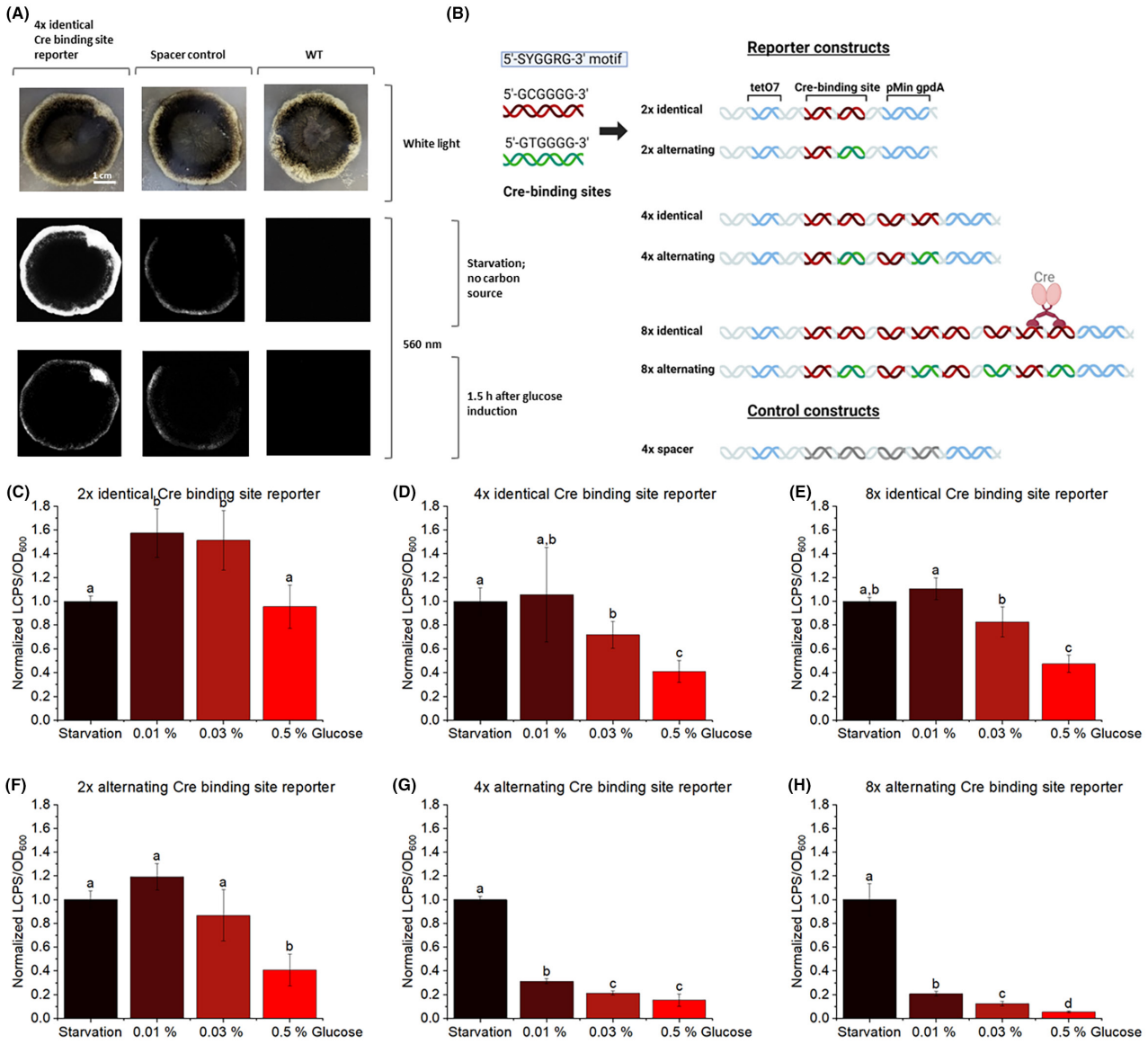

FIGURE 1 Construction and comparison of Cre-dependent CCR reporter with different numbers and types of Cre-binding sites in *Aspergillus niger*. A macroscopic luciferase assay was performed for biomass grown on solid medium. The 4× identical Cre-binding site luciferase reporter construct as well as the spacer construct containing no Cre-binding sites and the WT strain for control were compared in their luminescence emissions upon 50% glucose induction. (A) Pictures of the luminescence signal were taken after 2.5 h of induction with doxycycline and D-luciferin in aMM without carbon source and 1.5 h after glucose induction. (B) Illustration depicts the constructed variants of the Cre reporter (created with BioRender.com) tested in (A). Luciferase microtiter plate assay was performed for six different luciferase reporter constructs (C–H) with 2, 4 or 8 alternating and identical Cre-binding sites. After 6 h of induction with doxycycline and D-luciferin in aMM without carbon source, different concentrations of glucose (0.01%, 0.03% and 0.5%) were added. Luminescence values were measured after 3.3 h of glucose induction and normalized to the luciferase value under starvation condition with 0.1% glycerol. The WT control strain was used as blank. Different letters indicate statistically significant differences according to an ANOVA test ($n = 3$), $p < 0.05$. The strains were tested by inoculation of one transformant in three biological replicates.

glucose. This response was not observed in the spacer sequence control reporter (Figure S3).

To assess the responsiveness to glucose-triggered carbon catabolite repression, the reporter strains were stimulated with glucose following a starvation period. *A. niger* was starved for 1.5 h, and *N. crassa* for 45 min prior to glucose addition, allowing for the accumulation of luciferase. After the starvation period, water or 0.5% glucose was added in the exponential phase of signal increase and the phenotypic reaction was observed in real time. An increase in signal was observed in both ascomycetes upon starvation (Figure 2A,B). In *N. crassa*, however, the luciferase output was found to decline again 1.5 h post-induction, reaching a steady-state after about 8 h.

The addition of glucose to the medium led to a stall in luminescence at very low intensities in a *A. niger* (Figure 2A). In *N. crassa*, the luciferase signal continued

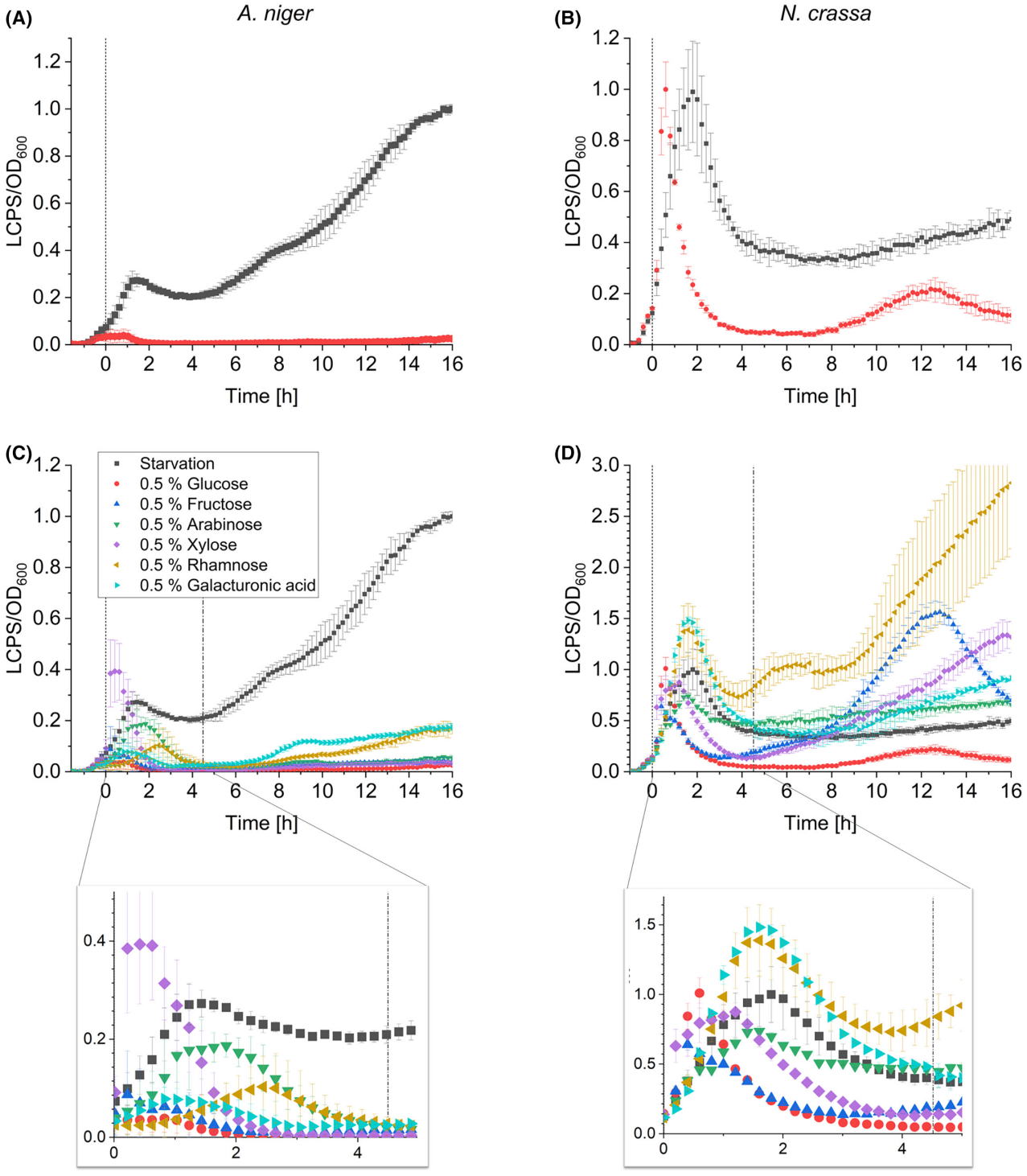

FIGURE 2 Comparison of *Aspergillus niger* and *Neurospora crassa* reporter strains upon different monosaccharide inductions. Luciferase microtiter plate assay was performed for *A. niger* (A) and *N. crassa* (B) transformed with the 8× alternating Cre-binding site reporter upon starvation and induction with 0.5% glucose. The assay was also performed for *A. niger* (C) and *N. crassa* (D) upon starvation and induction with 0.5% fructose, arabinose, xylose, rhamnose and galacturonic acid. The induction condition with 0.5% glucose was also included as a comparison. After 1.5 h/45 min of starvation in medium with doxycycline and D-luciferin in aMM without carbon source, different monosaccharides were added (dotted line; $t = 0$). Luminescence values were measured, normalized to the highest measured luciferase value under starvation conditions and blanked with the WT control strain ($n \geq 3$). The strains were tested by inoculation of one transformant in three biological replicates. The dash-dotted line at 4.5 h post-induction marks a point with relatively stable luminescence values.

to rise for 45 min after the glucose addition, leading to a peak of luciferase signal (Figure 2B). The same peak height was observed upon starvation but occurred 1.5 h later. It seems that glucose positively influenced luciferase production or created a more favourable environment for luciferase activity, likely due to higher ATP levels

in the cell initially, before inhibition took effect (Marques et al., 2009). Subsequently, after reaching a plateau, the signal in presence of glucose was only about 12.5% of the luminescence level, reached during starvation conditions. In *A. niger*, the luminescence dropped to 1.6% compared to starvation condition at 4.5 h after glucose addition, suggesting that the onset of CCR occurs more strongly at this glucose concentration than in *N. crassa*.

Comparative analysis of the CCR-inducing effect of different monosaccharides

In various studies, it was observed that monosaccharides other than glucose can also trigger CCR (Adnan et al., 2017; de Vries et al., 1999; Peng et al., 2021; Tamayo-Ramos et al., 2012). However, it has not been possible to evaluate quantitative and time-dependent effects of these monosaccharides and whether different filamentous fungi show variable Cre-mediated responsiveness on these with the common methods used to observe CCR. Thus, the reporter tool was utilized to examine the impact of various monosaccharides commonly found in plant cell wall material on Cre-mediated CCR in *A. niger* and *N. crassa*.

All monosaccharides were added to the cultures for a final concentration of 0.5%. This concentration was selected to maintain a high level of monosaccharides throughout the entire measurement, despite the fungus consuming some of it over time. 0.5% glucose led to the strongest and most rapid signal reduction in both ascomycetes (Figure 2C,D). Also, fructose, xylose, arabinose and galacturonic acid decreased the luminescence in both fungi. Intriguingly, rhamnose triggered CCR in *A. niger* but not in *N. crassa*, demonstrating that the sensitivity for some sugars differs between fungi. It is worth mentioning that in *N. crassa*, the luminescence output of strains grown in all carbohydrates except for glucose exceeded the starvation signal at some point in the run. Especially rhamnose, galacturonic acid and arabinose produced a higher signal for most time of the run, suggesting that a concentration of 0.5% of these carbon sources has a low impact on CCR but rather acts as an energy source, leading to a higher luciferase and ATP production and thus a stronger signal.

In *A. niger*, repression of luciferase synthesis occurred immediately after addition of glucose and fructose. For xylose, galacturonic acid, arabinose and rhamnose, the inhibition was delayed in the listed order, visible by a continued rise in luminescence prior to the drop of the light signal. The early luminescence maximum after addition of xylose exceeded the luciferase value of starvation at this time point by 2.6 times, but then dropped to 1.6% of the starvation signal level, equalling the signal in presence of glucose at 4.5 h post-addition (Table 1). At this time point, fructose reduced the signal to 6.9%, arabinose to 9.5%, rhamnose to 13.4% and galacturonic acid to 11.9% of the luciferase value in starvation condition, indicating that 0.5% glucose and xylose induce CreA-mediated CCR significantly stronger than the other monosaccharides ($p < 0.05$).

In *N. crassa*, all monosaccharides led to an elevated signal directly after their addition to the medium compared to the starvation condition, suggesting that the carbon sources are used as an energy source (at least briefly) before triggering CCR. Subsequently, glucose, fructose and xylose initiated signal inhibition first, reducing the output signal down to 12.5% for glucose, 46% for fructose and 35.3% for xylose 4.5 h after addition. At the same time point, the signals for arabinose, rhamnose and galacturonic acid were higher than in the starvation control condition, measured at 119%, 198% and 119%, respectively, demonstrating significant differences in luminescence repression between all monosaccharides at 0.5% concentration ($p < 0.01$; Table 1). The value during starvation differed from the arabinose-induced strain with lower observed statistical significance ($p < 0.05$).

At later time points, a release of Cre-mediated CCR could be observed in presence of several sugars in both fungi. While this was most prominent for rhamnose, fructose and xylose in *N. crassa*, the effect was much weaker in *A. niger*, with only rhamnose and galacturonic acid showing signs of CCR release after about 8–9 h. Overall, it could be seen that besides glucose, other carbon sources are able to trigger Cre-mediated CCR. However, the patterns of intensity and duration vary significantly, likely due to differences in uptake and consumption among the tested carbon sources and fungi.

Derepression of CCR by CreA truncations and deletion

To reduce CCR in biotechnologically used ascomycetes, Cre is often engineered. In *A. nidulans*, this was done by truncating CreA (Ries et al., 2016; Shroff et al., 1996). In this work, the approach was transferred

TABLE 1 OD_{600}-normalized LCPS values after 4.5 h of induction with 0.5% of various carbon sources normalized to starvation condition at 4.5 h in percentage.

Ascomycete	Starvation	Glucose	Fructose	Arabinose	Xylose	Rhamnose	Galacturonic acid
Aspergillus niger	100 ± 1	1.6 ± 0.07	6.9 ± 0.4	9.5 ± 0.8	1.6 ± 0.2	13 ± 1	11.9 ± 0.2
Neurospora crassa	100 ± 5	12.5 ± 0.9	46 ± 3	116 ± 2	35.3 ± 0.2	198 ± 17	119 ± 3

into *A. niger* to generate four strains harbouring different CreA truncations (Figure 3A).

The truncation strains were phenotypically examined on aMM plates with 2% glucose and plates with 1% xylose (Figure 3B and Table S3). Compared to the wild type (WT), all CreA truncations affected growth speed and led to a smaller growth diameter, proportional to the size of the truncation. After 7 days, the growth of the strain CreA(1–345) was reduced by 16%; for CreA(1–281), a reduction of 34%; and CreA(1–162), CreA (1–115) and the full deletion strain Δ*creA*, a reduction around 45% compared to the WT strain was visible (Table S3). The CreA truncation strains additionally displayed a successively reduced formation of black conidia after 4 days of inoculation. Conidiation seemed to be slower in CreA(1–281) and CreA(1–162), while for CreA(1–115) and Δ*creA*, even after 4 days, nearly no black-coloured conidia were visible.

To evaluate CCR derepression of the CreA truncations strains, 2-DG plate assays were performed (Figure 3C). All tested strains except the full deletion strain Δ*creA* and the strain with the smallest remaining construct, CreA(1–115), produced smaller colony diameters upon incubation with 2-DG on aMM + 1% xylose, indicating CCR derepression in these strains. Generally, the repressive effect of 2-DG was inversely correlated with the length of the CreA truncation. Surprisingly, the colony diameter of Δ*creA* on 2-DG was also reduced by 5%, which was significantly more (at $p < 0.05$) than for the CreA(1–115) strain.

To assess the CreA truncation-dependent derepression in a quantifiable real-time approach, the truncation strains were transformed with the 8× alternating Cre-binding site luciferase reporter and tested upon addition of 0.5% glucose.

As previously shown, the WT strain transcribing the full-length CreA variant was strongly inhibited, showing a fast signal decrease within less than 1 h of glucose addition (Figure 3D). The same was observed for the version containing CreA(1–345), although the signal of the starvation luminescence became increasingly noisy (Figure 3E). This strain depicted repression of 92.5% compared to 98.3% in the WT strain at 4.5 h after glucose addition compared to starvation ($p < 0.01$, $n = 3$). After 4.5 h of glucose addition, the strain carrying the truncated CreA(1–281) version produced a luciferase output signal that was not significantly different ($p > 0.05$) compared to starvation (Figure 3F). In this time course, truncation strains with CreA versions equal to or shorter than 162 aa even depicted higher luminescence values in glucose probably due to higher energy intake combined with low or abolished responsiveness of CreA-dependent CCR (Figure 3G,H). Unexpectedly, the Δ*creA* strain emitted a 27.2% lower luminescence in glucose compared to starvation ($p < 0.01$), but still exerted a clear CreA-mediated derepression compared to the WT strain. In the later course (10 h post-induction), the luciferase signal in the strain induced with glucose was much higher compared to the starvation condition (Figure 3I). A decline of luminescence over time could be explained by phenotypic side-effects of this mutation influencing metabolism, transcription and ATP availability for the luciferase reaction (Marques et al., 2009; Rassinger et al., 2018; Robl et al., 2018; Shroff et al., 1996, 1997). The truncations lacking the conserved region produced luminescence signals comparable to the starvation condition after 0.5% glucose addition, leading us to assume that the conserved domain might be essential for CreA-mediated gene repression in *A. niger*. To test this, a specific deletion of this domain was generated [CreA × (Δ281–345)] and found to lead to a strong derepression in presence of 0.5% glucose as well (Figure 4), which was clearly visible despite a slightly altered luminescence signal in absence of glucose (starvation) for this mutant strain.

Based on the observed derepression in 0.5% glucose, we conclude that the conserved domain (between amino acids 281 and 345), and not the repressing region at the C-terminus, is essential for functioning of CreA as transcriptional repressor in *A. niger*. As an additional verification for the *creA* deletion strains, we also compared the production of hydrolytic enzymes in CreA(1–281), CreA(1–162) and Δ*creA* to the WT. Especially, the strain CreA(1–281) showed the highest enzymatic activity in all performed assays (Figure S6).

Application of the reporter tool in *A. nidulans*

From an early stage, *A. nidulans* was utilized as a reference organism for studying CCR in filamentous fungi (Bailey & Arst, 1975). Since much of our current understanding of CreA-mediated CCR was developed in *A. nidulans* (de Assis et al., 2018, 2021; Ries et al., 2016), providing an applicable CCR reporter tool could further support these efforts. We therefore wanted to test whether our construct would also work in this important reference system. To this end, *A. nidulans* (GR5) was transformed with the 8× alternating binding site reporter and the spacer control construct using a transferable system for ectopic integration. We tested several transformants to evaluate the responsiveness of the reporter to 0.5% glucose. After 4.5 h post-induction, stable luminescence values were measured. A drop in luciferase signal was measurable after glucose addition in the tested reporter strains. In contrast, the strains transformed with the spacer construct without Cre-binding site were unaffected by CCR and produced equal or even higher light output upon glucose addition (Figure 5), thus proving the functionality of the reporter system also in *A. nidulans*.

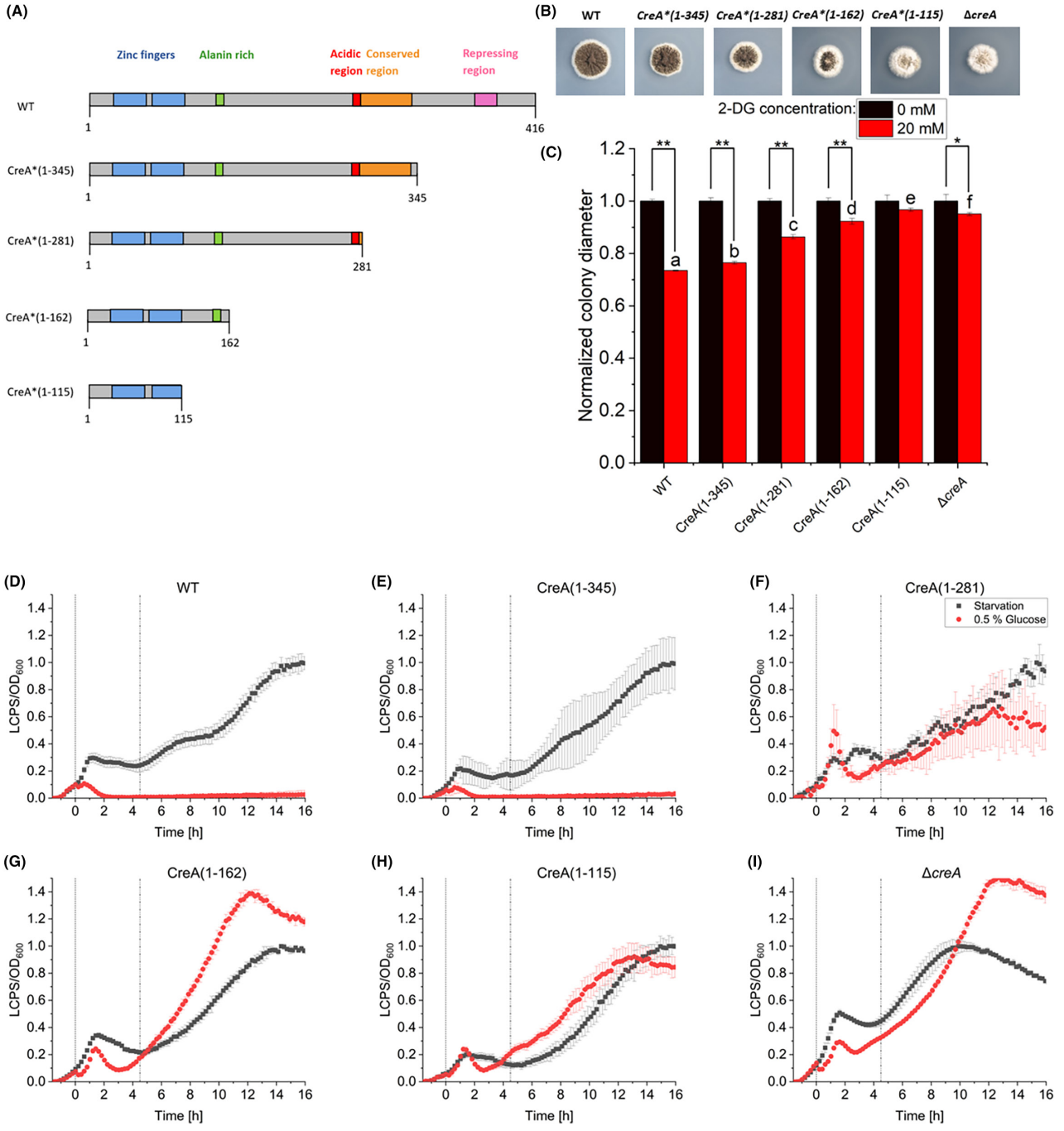

FIGURE 3 Phenotypic observation, 2-DG and CreA luciferase reporter assay comparing *Aspergillus niger* truncation strains. Comparison of CreA truncation constructs is depicted in (A). Growth and sporulation of *A. niger* WT, CreA truncation strains CreA(1–345), CreA(1–281), CreA(1–126), CreA(1–115) and Δ*creA* on aMM + 2% glucose were compared after 4 days of incubation at 30°C (B). Different letters indicate statistically significant differences according to an ANOVA test ($n = 3$), $p < 0.05$. Strains were generated by CRISPR/Cas9-mediated truncations of the native *CreA* locus, and three biological replicates per strain were tested. Growth diameter of *A. niger* WT, CreA truncation strains CreA(1–345), CreA(1–281), CreA(1–126), CreA(1–115) and Δ*creA* were compared upon CCR induction with 20 mM 2-DG on 1% xylose after 7 days of incubation at 30°C (C). The diameters of three colonies per strain ($n = 3$) were measured and the average was calculated. Luciferase microtiter plate assay was performed for *A. niger* WT, CreA truncation strains CreA(1–345), CreA(1–281), CreA(1–126), CreA(1–115) and Δ*creA* (D–I) transformed with the 8× alternating Cre-binding site reporter upon starvation and induction with 0.5% glucose. After 1.5 h of induction with doxycycline and D-luciferin in aMM without carbon source, 0.5% of glucose was added (dotted line). Luminescence values were measured, normalized to the highest luciferase value under starvation conditions and blanked with the WT control strain ($n \geq 3$). The strains were tested by inoculation of one transformant in three biological replicates. The dash-dotted line at 4.5 h post-induction marks a point with relatively stable luminescence values.

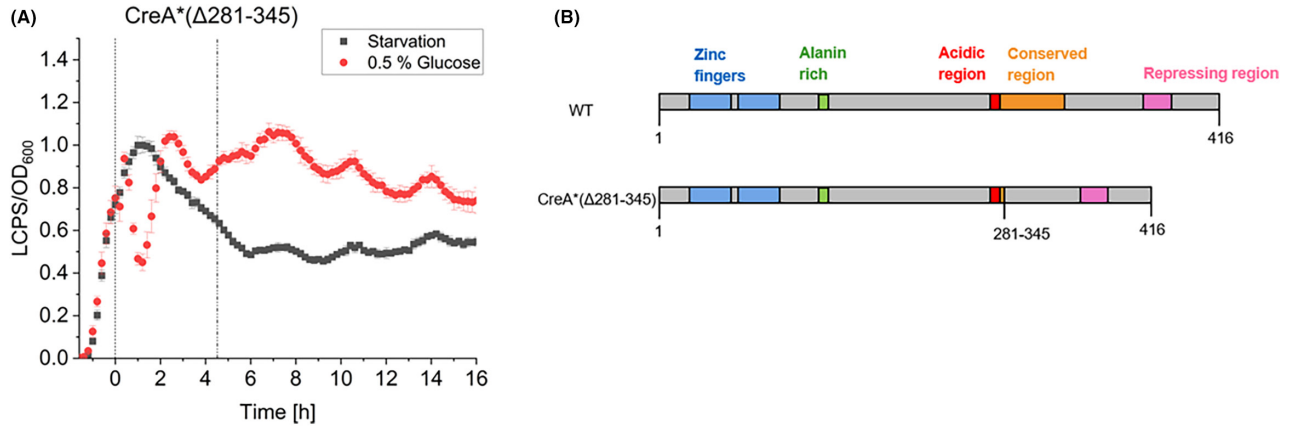

FIGURE 4 CreA luciferase reporter assay in the strain carrying the deletion of the conserved region in CreA. Luciferase microtiter plate assay was performed for *Aspergillus niger* CreA × (Δ281–345) deletion strain (A) transformed with the 8× alternating Cre-binding site reporter upon starvation and induction with 0.5% glucose. After 1.5 h of starvation in medium with doxycycline and D-luciferin in aMM without carbon source, 0.5% of glucose was added (dotted line). Luminescence values were measured, normalized to the highest luciferase value under starvation condition and blanked with the WT control strain ($n \geq 3$). The strains were tested by inoculation of one transformant in three biological replicates. The dash-dotted line at 4.5 h post-induction marks a point with relatively stable luminescence values. Schematic representation of the CreA deletion strain [CreA × (Δ281–345)] (B). The strain was generated upon CRISPR/Cas9-mediated deletion of the conserved region.

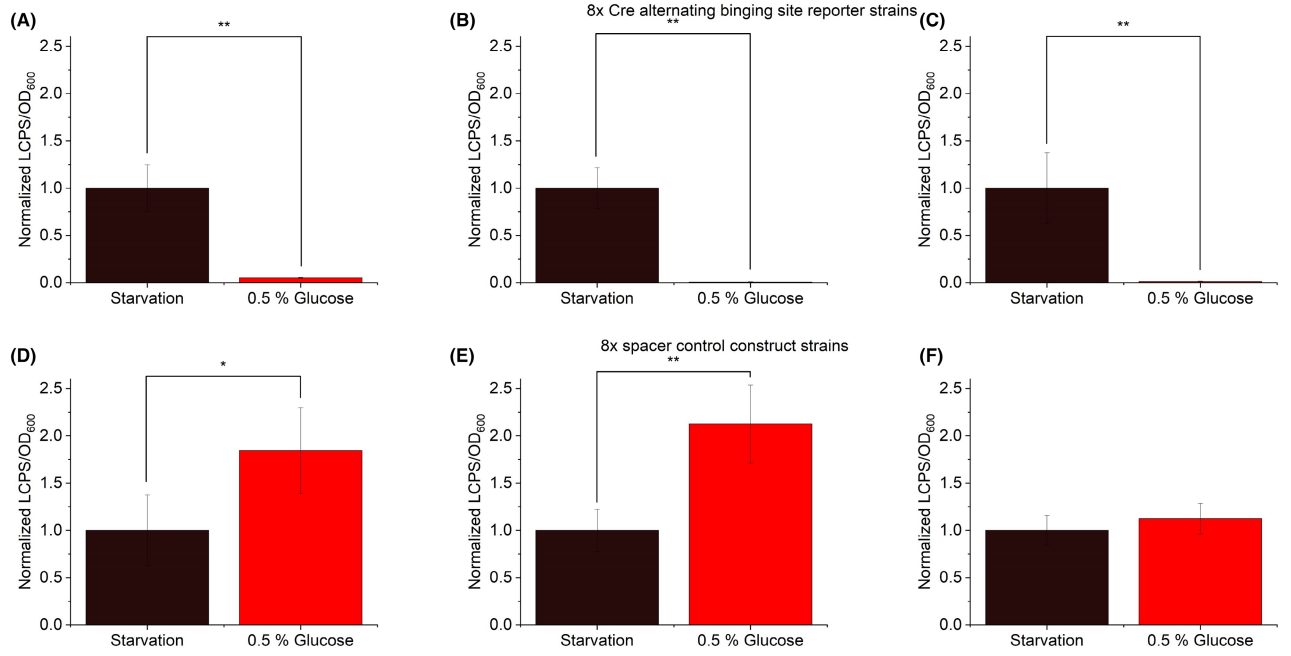

FIGURE 5 Comparison of *Aspergillus nidulans* reporter and control strain upon glucose induction. Luciferase microtiter plate assay was performed for three replicates of *A. nidulans* strains transformed with the 8× alternating Cre-binding site reporter (A–C) and the control with a spacer sequence (D–F) upon starvation and induction with 0.5% glucose. After 1.5 h of starvation in medium with doxycycline and D-luciferin in aMM without carbon source, 0.5% of glucose was added. Luminescence values were measured 4.5 h after induction, normalized to the luciferase value under starvation conditions and blanked with the WT control strain ($n \geq 3$). Three transformants per construct (A–C, D–F) were tested by inoculation in three biological replicates each. * and ** indicate statistically significant differences according to an ANOVA test, $p < 0.05$; $p < 0.01$.

DISCUSSION

By integrating Cre-binding sites into a doxycycline-inducible promoter of a luciferase reporter cassette, we were able to establish a transferable real-time tool to quantify Cre-mediated CCR at the macroscopic level. This can help to understand carbon regulatory processes of fungi in plate and liquid cultures.

To find an optimally responding system, two Cre-binding site variants were tested in 2, 4 and 8 identical or alternating repeats by integration into the reporter's promoter. Our results showed that

alternating 5′-GTGGGG-3′ and 5′-GCGGGG-3′ sites resulted in more sensitive and tighter controlled reporters than those constructs containing identical 5′-GTGGGG-3′ repeats. These results indicate that in *A. niger*, CreA binding to the 5′-GCGGGG-3′ site present in the alternating binding site reporter may be stronger than to the 5′-GTGGGG-3′ version. Both binding sites used contain the 5′-SYGGRG-3′ motif described to be bound by Cre in various fungi and both motifs are strongly bound by CRE-1 in *N. crassa*, as shown by chromatin immunoprecipitation (Espeso & Peñalva, 1994; Kulmburg et al., 1993; Sun & Glass, 2011). Usage of multiple binding site variants could therefore improve the chances of strong inhibition even when one of the binding sites is bound less efficiently, and thus could ensure transferability to other filamentous fungi, which was verified for *A. nidulans* and *N. crassa* so far. In some cases, however, it might even be helpful to use a reporter with lower stringency to quantify CCR under highly repressive conditions like high-glucose concentrations, broadening the usability of the Cre reporter toolkit.

In this work, the CCR effect of low-glucose concentrations and various monosaccharides was tested in *A. niger* and *N. crassa*, using the most responsive CCR reporter previously selected to quantify CCR effects. Using glucose concentrations in the range of 0.01–0.5%, higher concentrations led to stronger repression of luminescence. Upon starvation, the luciferase signal in *A. niger* was found to rise continuously over the course of the experiment, while it declined in *N. crassa* after 2.25 h to a lower steady-state level. It has been described that intracellular protein degradation is higher under nutritional stress (Martegani & Alberghina, 1979), and it seems likely that the ratio of luciferase production and degradation is dependent on the available energy for synthesis, which might have been different in the two fungi. Additionally, the luciferase reaction is highly dependent on ATP, leading to a decreased signal under low ATP conditions during starvation (Marques et al., 2009).

After glucose addition, the luciferase production was almost instantaneously slowed down in *A. niger*, while in the case of *N. crassa*, a signal peak was observed directly after glucose addition, exceeding the luminescence of the strains under starvation. Light output of the constructs in the fungi is likely a combined effect of all cellular processes affecting CreA activity, driven mainly by the intracellular concentrations of glucose or some metabolites downstream of the glucose catabolism, which itself is positively influenced by the uptake and the production of the CCR inducer molecule on the one hand, while a high catabolic rate of the inducer negatively influences CCR. These observations are in line with a higher internal carbon availability at the start of the assay in *A. niger* compared to *N. crassa*. Intracellular concentrations of the CCR inducer in *N. crassa* were initially likely below the threshold to trigger Cre-mediated CCR due to a low amount of stored carbon, combined with higher catabolic rates compared to sugar uptake. The energy derived from fast glucose catabolism could explain why the light signal was seen to increase faster than under starvation conditions. After 45 min of glucose addition, the luminescence then decreased rapidly, indicating that the intercellular glucose concentration reached a level above the threshold for CCR.

While analyses of CCR are most commonly performed with glucose, it was already shown, for example, in *A. niger* that CreA-mediated CCR is substrate dependent and that other carbon sources can trigger CCR as well (de Vries et al., 1999; Peng et al., 2021). However, so far it has been difficult to directly compare the CCR-inducing effect of different sugars in a quantitative fashion or with a high temporal resolution. This was evaluated with the new reporter construct in this work.

In *A. niger*, all tested sugars caused CreA-mediated repression, albeit with a different timing. An immediate repressive effect was observed after addition of glucose and fructose in contrast with xylose, galacturonic acid, arabinose and rhamnose, which led to a somewhat delayed inhibition (in that order). In the case of xylose, also a strong signal peak was measured following addition to the culture. It seems that for these carbon sources, the threshold concentration for the specific inducer was not reached during the first 30 min post-addition. Later on, however, higher internal concentrations of CCR-inducing molecule(s) are reached, leading to signal loss. In *S. cerevisiae*, it was described that fructose-6-phosphate (F6P) and cyclic adenosine monophosphate (cAMP) set off CCR. Besides these, it is not known whether other molecules can have the same effect (Gancedo, 1998). However, due to the interconnections between the different monosaccharide catabolic pathways, it could be that an increase in F6P and cAMP is eventually occurring for all sugars. In case of the pentoses (xylose and arabinose), for example, their metabolism is linked to the glycolysis from the non-oxidative branch of the pentose phosphate pathway via the transketolase reaction of D-xylulose-5-phosphate (X5P) to F6P (Masi et al., 2021). Arabinose needs five reactions to be transformed to D-xylulose-5-phosphate, including the oxidation from arabitol to L-xylulose, which is considered a bottleneck in this pathway (Chroumpi et al., 2021; Groot et al., 2005). Xylose, on the other hand, only needs three reactions, which is in line with a more rapid triggering of CCR compared to arabinose. Similar metabolic effects could also be responsible for the delay in response of the other different monosaccharides. It is therefore intriguing to hypothesize that the delay of the onset of CCR as measured with the reporter tool is representative of the speed of sugar metabolism (including their uptake) until the catabolic

products result in a substantial increase of glycolytic intermediates, in particular F6P, which would eventually trigger CCR. Similarly, longer (and/or slower) reaction pathways have often been seen to lead to delayed responses (Li et al., 2022).

At 4.5 h post-induction, stable luciferase signals were reached in the different carbon sources, suggesting balanced intracellular concentrations at this time. After 4.5 h, a great reduction in signal was measured for glucose, xylose and fructose and a weaker decrease for arabinose, rhamnose and galacturonic acid compared to the values under starvation. In addition, these three monosaccharides triggered the decrease in luciferase more rapidly after induction of CCR. Prathumpai et al. (2004) found that in *A. nidulans*, glucose was catabolized prior to xylose, mediated by CreA, supporting the hypothesis that 0.5% xylose is a less efficient CCR inducer than glucose (Prathumpai et al., 2004). Furthermore, the breakdown of storage carbohydrates could influence CCR and therefore the luciferase reporter output signal, especially at later time points.

It was observed that the rhamnose catabolism in *A. nidulans* is inhibited by arabinose in a CreA-dependent manner, suggesting that the CCR effect of arabinose is stronger compared to the effect of rhamnose (Tamayo-Ramos et al., 2012). This could be shown for *A. niger* in the performed assay by a weaker reduction in light emission upon rhamnose addition compared to arabinose after 4.5 h of induction. Moreover, the CCR effect of galacturonic acid had previously been described as much lower compared to glucose (de Vries et al., 1999; Peng et al., 2021). This was similar to what we could observe, particularly considering that the repression by galacturonic acid, similar to rhamnose, was found to diminish already after 6.5 h while lasting much longer for glucose and the other sugars leading to raising luminescence values.

In case of *N. crassa*, a more prominent increase in signal intensity was observed after addition of all the different carbon sources, presumably due to the release of nutritional stress. Also here, the timing until a decrease in signal was measured differed between the sugars, ranging from 1.25 h for glucose and fructose to 1.5 h for xylose and 2.25 h for arabinose, rhamnose and galacturonic acid. A faster accumulation of the molecules that lead to CCR or a varying threshold for these could explain differences in triggering CCR between various carbon sources. After 4.5 h, relatively stable values became apparent with rather strong signal reduction for glucose, fructose and xylose and an increased signal for arabinose, rhamnose and galacturonic acid compared to the no carbon control. In both fungi, glucose, fructose and xylose therefore showed strong CCR effects at this time point. However, while arabinose, rhamnose and galacturonic acid reduced the luminescence in *A. niger*, this did not happen the same way in *N. crassa* 4.5 h post-induction. In *A. niger* 0.5% galacturonic acid led to the lowest luciferase inhibition compared to starvation, while in *N. crassa* rhamnose addition generated the highest values.

In summary, the distinct responses of *A. niger* and *N. crassa* to diverse carbon sources exhibited variations in intensity and temporal dynamics. This underscores the utility of the engineered reporter tool in elucidating physiological distinctions and depicting disparate inhibition patterns following fungal exposure to carbon sources. Such insights were previously unattainable with traditional methodologies.

Upon using the CreA truncation strains in a luciferase reporter assay, we were able to obtain a derepression pattern similar to that of the 2-DG assay. Strains carrying the full-length or the longest version of CreA [CreA(1–345)] were strongly inhibited by glucose in the luciferase reporter assay and also showed growth inhibition in the 2-DG assay. In aMM with 2% of glucose, only a slight colony reduction was observed. Deletion of the 'repression region' in the C terminus of CreA was previously used in *A. nidulans* to study the influence of the domain on binding to the promoter of the CCR-repressed *xlnA* gene (Ries et al., 2016). Upon performing ChIP–qRT-PCR, it was demonstrated that this deletion prevents the repressor from binding to this promoter region. Another study demonstrated that important regulator recognition sites that influence activity and cellular transport can be found in this region (Tanco et al., 2015), explaining the observed derepression phenotype. It seems that in *A. niger*, however, the deletion of this site is not sufficient to abolish CreA-mediated CCR and thus the ability of CreA to bind to DNA.

The strain carrying the CreA(1–281) truncation with a deletion of the conserved region was more strongly released from repression in the 2-DG assay and in the quantitative luciferase reporter assay. This strain did not exhibit repression upon 0.5% glucose and showed no difference in luminescence upon starvation and glucose induction. In the 2-DG assay, a decrease in colony size was still measurable, although the size difference was smaller compared to the WT and the CreA(1–345) strain. This truncation reduced the colony size on aMM with 2% glucose and slightly delayed sporulation. According to Shroff et al. (1996), *A. nidulans* strains expressing the CreA variant without this region display higher sensitivity to allyl alcohol in the presence of glucose than the wild-type, while showing lower growth defects (Shroff et al., 1996), indicating efficient CCR derepression with only minor side-effects. This region is known to harbour many phosphorylation sites important for the translocation to the nucleus and binding to CCR target genes upon glucose-mediated phosphorylation (de Assis et al., 2021). The conserved domain therefore seems to be important for the functionality of CreA, and this assumption was supported by our data, which suggest it to be essential for the binding to its regulon.

De Assis also predicted many phosphorylation sites in the acidic region of CreA from *A. nidulans* (de Assis et al., 2021). In *T. reesei* and *Sclerotinia sclerotiorum*, a phosphorylation site in the acidic region is essential for binding the Cre1 repressor to its genomic binding sites (Cziferszky et al., 2002; Vautard-Mey & Fèvre, 2000). This could explain why CreA(1–162) and CreA(1–115) truncation strains lacking the acidic region showed strong derepression in both assays. In the luciferase assay, CreA(1–162) and CreA(1–115) showed complete derepression by increased light emission in glucose compared to starvation and CreA(1–162) was strongly derepressed in the 2-DG assay. A point mutation of S262 in *A. nidulans*, which is equivalent to the phosphorylation site in *T. reesei* and *S. sclerotiorum*, did not affect the binding of the mutated repressor but decreased its abundance, which consequently led to higher hydrolase production (de Assis et al., 2021). Since CCR was observed in corresponding *A. nidulans* mutants lacking the acidic domain, this deletion seems to not be sufficient for complete derepression. Considering its close proximity to the conserved region, the acidic domain could contribute to its function, which would explain why deletion of both domains led to severe derepression in the luciferase-based CCR reporter assay [CreA(1–162)]. In *Metarhizium acridum*, *T. reesei* and *A. nidulans*, a homologous truncation of Cre led to a complete derepression (Nakari-Setälä et al., 2009; Song et al., 2019; Zhang, Wu et al., 2018; Zhang, Zhang et al., 2018). Although CreA(1–162) and CreA(1–115) showed higher luminescence upon 0.5% glucose induction in the luciferase reporter assay, only the CreA(1–115) truncation was completely derepressed in the 2-DG assay, showing no significant growth reduction with 2-DG. In this truncation strain, the growth was not additionally reduced compared to CreA(1–162), but a delay in sporulation was observed, comparable to the phenotype of Δ*creA*. A single deletion of the alanine-rich domain in *A. nidulans* did not reduce growth, suggesting that this region is not essentially involved in colony growth on glucose (Ries et al., 2016). It is hypothesized that the CreA(1–115) truncation can no longer exhibit gene repression, and in another study, it was even reported that the homologous truncation in *T. reesei* RUT-C30 acts as a transcriptional activator (Eveleigh & Montenecourt, 1979; Mello-de-Sousa et al., 2014; Rassinger et al., 2018).

In the 2-DG and also in the Cre luciferase reporter assays, Δ*creA* led to increased repression compared to CreA(1–115), providing evidence that other negative transcriptional regulators could be blocked by CreA(1–115), leading to a more CCR-unaffected strain compared to the other strains, including Δ*creA*. In *S. cerevisiae* and *A. nidulans*, multiple transcription factors were found to act together to inhibit glucose-repressed genes that bind to similar binding sites (Alam & Kelly, 2017; Westholm et al., 2008). Furthermore, as shown in other organisms, knocking out CreA generally leads to different phenotypes, which are also visible in the *A. niger* Δ*creA* KO strain (Rassinger et al., 2018; Robl et al., 2018; Shroff et al., 1996, 1997). In our study, this strain showed smaller colony diameters and delayed conidiation compared to the WT strain. To sum up, the decrease in Cre-based inhibition seems to be achieved by a trade-off with impaired growth and sporulation. CreA truncation mutants show similar levels of CCR derepression compared to 2-DG assays but in a better quantifiable and real-time approach. The strains derepressed in 0.5% glucose [CreA(1–281) and CreA(1162)] with the lowest phenotypic side-effects also showed the highest hydrolase production in medium containing hemicellulose and glucose (Figure S6). Furthermore, the dynamics of the CCR effect of various monosaccharides in two filamentous fungi was studied using the developed tool. The gradual removal of the domains depicts that each region contributes to the functionality of Cre. However, the strongest derepression was observed in our assays when the conserved region was removed.

CONCLUSION

Due to its central role in production of hydrolases and secondary metabolites but also regulation of pathogenicity and symbiosis, CCR needs to be studied well from various angles. In this work, we were able to create a real-time tool for quantification of Cre-mediated CCR effects of gene repression, using a luciferase-based reporter. By varying number and type of Cre-binding motifs, reporters with different stringency were established. The most sensitive reporter containing 8× alternating Cre-binding repeats of 5′-GTGGGG-3′ and 5′-GCGGGG-3′ was proven to work in *A. niger*, *N. crassa* and *A. nidulans*. The luciferase output was detectable not only on microtiter level but also on macroscopic level, broadening the applicability to study CCR effects of fungal colonies. This could unravel new insights in carbon-regulated processes like pathogenicity or symbiosis. By quantifying the effects of different carbon sources on Cre-mediated CCR, clear differences were observed for *A. niger* and *N. crassa*, demonstrating that the new reporter tool can contribute to better understanding CCR-related physiological processes of different filamentous fungi and thus be used to screen for optimized industrial fungal fermentation conditions to generate higher yields. For example, it could be used to effectively identify the optimal timing for omics experiments, such as transcriptomics, proteomics and metabolomics, to ensure a better correlation between the intracellular metabolic state and the observed response. By aligning the timing of these experiments with critical cellular events, researchers

can gain deeper insights into the dynamic processes within cells, leading to more meaningful and comprehensive data. Moreover, as demonstrated in this work, this novel tool can be applied to test Cre mutant strains in a fast and quantitative manner to find CCR-derepressed strains for biotechnological applications. The straightforward design of the reporter highlights its potential for facile customization, enabling the construction of diverse variants tailored to a spectrum of applications. For example, by harbouring a selectable marker instead of luciferase, which could be used to screen for CCR mutants in a fast and reliable manner, due to the tight repression profile of the reporter. The reporter consequently offers versatility in addressing fundamental research questions, such as assessing the responsiveness of different fungi to CCR or studying the essential domains of Cre involved in CCR while also serving as a valuable tool for screening more efficient strains suitable for industrial applications.

AUTHOR CONTRIBUTIONS

Marcel Rüllke: Investigation; methodology; validation; visualization; formal analysis; writing – original draft; conceptualization; supervision. **Franziska Meyer:** Investigation; writing – review and editing; methodology; formal analysis. **Kevin Schmitz:** Conceptualization; writing – review and editing; methodology; investigation; formal analysis. **Hannes Blase:** Investigation; writing – review and editing; formal analysis. **Elisabeth Tamayo:** Writing – review and editing; supervision; conceptualization; validation. **J. Philipp Benz:** Conceptualization; writing – review and editing; project administration; supervision; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests to declare that are relevant to the content of this article.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article and its supplementary information files or can be obtained from the corresponding author upon reasonable request.

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REFERENCES

Adnan, M., Zheng, W., Islam, W., Arif, M., Abubakar, Y.S., Wang, Z. et al. (2017) Carbon catabolite repression in filamentous fungi. *International Journal of Molecular Sciences*, 19(1), 48. Available from: https://doi.org/10.3390/ijms19010048

Alam, M.A., Kamlangdee, N. & Kelly, J. (2016) The CreB deubiquitinating enzyme does not directly target the CreA repressor protein in *Aspergillus nidulans*. *Current Genetics*, 63, 647–667. Available from: https://doi.org/10.1007/s00294-016-0643-x

Alam, M.A. & Kelly, J.M. (2017) Proteins interacting with CreA and CreB in the carbon catabolite repression network in *Aspergillus nidulans*. *Current Genetics*, 63(4), 669–683. Available from: https://doi.org/10.1007/s00294-016-0667-2

Arentshorst, M., Ram, A.F.J. & Meyer, V. (2012) Using non-homologous end-joining-deficient strains for functional gene analyses in filamentous fungi. *Methods in Molecular Biology*, 835, 133–150. Available from: https://doi.org/10.1007/978-1-61,779-501-5_9

Aristidou, A. & Penttilä, M. (2000) Metabolic engineering applications to renewable resource utilization. *Current Opinion in Biotechnology*, 11(2), 187–198. Available from: https://doi.org/10.1016/s0958-1669(00)00085-9

Bailey, C. & Arst, H.N. (1975) Carbon catabolite repression in *Aspergillus nidulans*. *European Journal of Biochemistry*, 51(2), 573–577. Available from: https://doi.org/10.1111/j.1432-1033.1975.tb03958.x

Baxi, S.N., Portnoy, J.M., Larenas-Linnemann, D. & Phipatanakul, W. (2016) Exposure and health effects of fungi on humans. *The Journal of Allergy and Clinical Immunology: In Practice*, 4(3), 396–404. Available from: https://doi.org/10.1016/j.jaip.2016.01.008

Brown, N., De Gouvea, P.F., Krohn, N.G., Savoldi, M. & Goldman, G.H. (2013) Functional characterisation of the non-essential protein kinases and phosphatases regulating *Aspergillus nidulans* hydrolytic enzyme production. *Biotechnology for Biofuels*, 6(1), 91. Available from: https://doi.org/10.1186/1754-6834-6-91

Cesbron, F., Brunner, M. & Diernfellner, A.C.R. (2013) Light-dependent and circadian transcription dynamics in vivo recorded with a destabilized luciferase reporter in Neurospora. *PLoS One*, 8(12), e83660. Available from: https://doi.org/10.1371/journal.pone.0083660

Chakraborty, B.N. & Kapoor, M. (1990) Transformation of filamentous fungi by electroporation. *Nucleic Acids Research*, 18(22), 6737. Available from: https://doi.org/10.1093/nar/18.22.6737

Chroumpi, T., Peng, M., Markillie, L., Mitchell, H.D., Nicora, C.D., Hutchinson, C.M. et al. (2021) Re-routing of sugar catabolism provides a better insight into fungal flexibility in using plant biomass-derived monomers as substrates. *Frontiers in Bioengineering and Biotechnology*, 9, 644216. Available from: https://doi.org/10.3389/fbioe.2021.644216

Cziferszky, A., Mach, R.L. & Kubicek, C.P. (2002) Phosphorylation positively regulates DNA binding of the carbon catabolite

repressor Cre1 of *Hypocrea jecorina* (*Trichoderma reesei*). *The Journal of Biological Chemistry*, 277(17), 14688–14694. Available from: https://doi.org/10.1074/jbc.M200744200

de Assis, L.J., Silva, L.P., Bayram, O., Dowling, P., Kniemeyer, O., Krüger, T. et al. (2021) Carbon catabolite repression in filamentous fungi is regulated by phosphorylation of the transcription factor CreA. *MBio*, 12(1), 128. Available from: https://doi.org/10.1128/mBio.03146-20

de Assis, L.J., Ulas, M., Ries, L.N.A., El Ramli, N.A.M., Sarikaya-Bayram, O., Braus, G.H. et al. (2018) Regulation of *Aspergillus nidulans* CreA-mediated catabolite repression by the F-box proteins Fbx23 and Fbx47. *MBio*, 9(3), 1128. Available from: https://doi.org/10.1128/mBio.00840-18

de Vries, R.P., Visser, J. & de Graaff, L.H. (1999) CreA modulates the XlnR-induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation. *Research in Microbiology*, 150(4), 281–285. Available from: https://doi.org/10.1016/s0923-2508(99)80053-9

Dowzer, C.E. & Kelly, J.M. (1989) Cloning of the creA gene from *Aspergillus nidulans*: a gene involved in carbon catabolite repression. *Current Genetics*, 15(6), 457–459. Available from: https://doi.org/10.1007/BF00376804

Dowzer, C.E. & Kelly, J.M. (1991) Analysis of the creA gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Molecular and Cellular Biology*, 11(11), 5701–5709. Available from: https://doi.org/10.1128/mcb.11.11.5701-5709.1991

Ebbole, D.J. & Sachs, M.S. (1990) A rapid and simple method for isolation of *Neurospora crassa* homokaryons using microconidia. *Fungal Genetics Reports*, 37(1), 1472. Available from: https://doi.org/10.4148/1941-4765.1472

Espeso, E.A. & Peñalva, M.A. (1994) In vitro binding of the two-finger repressor CreA to several consensus and non-consensus sites at the ipnA upstream region is context dependent. *In FEBS Letters*, 342(1), 43–48. Available from: https://doi.org/10.1016/0014-5793(94)80581-4

Eveleigh, D.E. & Montenecourt, B.S. (1979) Increasing yields of extracellular enzymes. *Advances in Applied Microbiology*, 25, 57–74. Available from: https://doi.org/10.1016/s0065-2164(08)70146-1

Fellbaum, C.R., Gachomo, E.W., Beesetty, Y., Choudhari, S., Strahan, G.D., Pfeffer, P.E. et al. (2012) Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, 109(7), 2666–2671. Available from: https://doi.org/10.1073/pnas.1118650109

Franzino, T., Boubakri, H., Cernava, T., Abrouk, D., Achouak, W., Reverchon, S. et al. (2022) Implications of carbon catabolite repression for plant–microbe interactions. *Plant Communications*, 3(2), 100272. Available from: https://doi.org/10.1016/j.xplc.2021.100272

Gancedo, J.M. (1998) Yeast carbon catabolite repression. *Microbiology and Molecular Biology Reviews*, 62(2), 334–361. Available from: https://doi.org/10.1128/MMBR.62.2.334-361.1998

Groot, M.J.L., Prathumpai, W., Visser, J. & Ruijter, G.J. (2005) Metabolic control analysis of *Aspergillus niger* L-arabinose catabolism. *Biotechnology Progress*, 21(6), 1610–1616. Available from: https://doi.org/10.1021/bp050189o

Hicks, J., Lockington, R.A., Strauss, J., Dieringer, D., Kubicek, C.P., Kelly, J. et al. (2001) RcoA has pleiotropic effects on *Aspergillus nidulans* cellular development. *Molecular Microbiology*, 39(6), 1482–1493. Available from: https://doi.org/10.1046/j.1365-2958.2001.02332.x

Knesebeck, M., Schäfer, D., Schmitz, K., Rüllke, M., Benz, J. & Weuster-Botz, D. (2023) Enzymatic one-pot hydrolysis of extracted sugar beet press pulp after solid-state fermentation with an engineered *Aspergillus niger* strain. *Fermentation*, 9(7), 582. Available from: https://doi.org/10.3390/fermentation9070582

Kowalczyk, J.E., Benoit, I. & Vries, R.P. (2014) Regulation of plant biomass utilization in aspergillus. *Advances in Applied Microbiology*, 88, 31–56.

Kulmburg, P., Mathieu, M., Dowzer, C., Kelly, J. & Felenbok, B. (1993) Specific binding sites in the alcR and alcA promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. *Molecular Microbiology*, 7(6), 847–857. Available from: https://doi.org/10.1111/j.1365-2958.1993.tb01175.x

Lee, M.E., DeLoache, W.C., Cervantes, B. & Dueber, J.E. (2015) A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synthetic Biology*, 4(9), 975–986. Available from: https://doi.org/10.1021/sb500366v

Leskinen, P., Virta, M. & Karp, M. (2003) One-step measurement of firefly luciferase activity in yeast. *Yeast (Chichester, England)*, 20(13), 1109–1113. Available from: https://doi.org/10.1002/yea.1024

Leynaud-Kieffer, L.M.C., Curran, S.C., Kim, I., Magnuson, J.K., Gladden, J.M., Baker, S.E. et al. (2019) A new approach to Cas9-based genome editing in *Aspergillus niger* that is precise, efficient and selectable. *PLoS One*, 14(1), e0210243. Available from: https://doi.org/10.1371/journal.pone.0210243

Li, J., Chroumpi, T., Garrigues, S., Kun, R.S., Meng, J., Salazar-Cerezo, S. et al. (2022) The sugar metabolic model of *Aspergillus niger* can only be reliably transferred to fungi of its phylum. *Journal of Fungi*, 8(12), 8. Available from: https://doi.org/10.3390/jof8121315

Lockington, R.A. & Kelly, J.M. (2001) Carbon catabolite repression in *Aspergillus nidulans* involves deubiquitination. *Molecular Microbiology*, 40(6), 1311–1321. Available from: https://doi.org/10.1046/j.1365-2958.2001.02474.x

Lucca, D. & Anthony, J. (2007) Harmful fungi in both agriculture and medicine. *Revista Iberoamericana de Micología*, 24(1), 3.

Luehrsen, K.R., Wet, J.R. & Walbot, V. (1992) Transient expression analysis in plants using firefly luciferase reporter gene. *Methods in Enzymology*, 216, 397–414. Available from: https://doi.org/10.1016/0076-6879(92)16037-K

Marques, S.M., Silva, D.E. & Joaquim, C.G. (2009) Firefly bioluminescence: a mechanistic approach of luciferase catalyzed reactions. *IUBMB Life*, 61(1), 6–17. Available from: https://doi.org/10.1002/iub.134

Martegani, E. & Alberghina, L. (1979) Intracellular protein degradation in *Neurospora crassa*. *The Journal of Biological Chemistry*, 254(15), 7047–7054.

Masi, A., Mach, R.L. & Mach-Aigner, A.R. (2021) The pentose phosphate pathway in industrially relevant fungi: crucial insights for bioprocessing. *European Journal of Applied Microbiology and Biotechnology*, 105(10), 4017–4031. Available from: https://doi.org/10.1007/s00253-021-11,314-x

Matar, K.A., Chen, X., Chen, D., Anjago, W.M., Norvienyeku, J., Lin, Y. et al. (2017) WD40-repeat protein MoCreC is essential for carbon repression and is involved in conidiation, growth and pathogenicity of *Magnaporthe oryzae*. *Current Genetics*, 63(4), 685–696. Available from: https://doi.org/10.1007/s00294-016-0668-1

Mello-de-Sousa, T.M., Gorsche, R., Rassinger, A., Poças-Fonseca, M.J., Mach, R.L. & Mach-Aigner, A.R. (2014) A truncated form of the carbon catabolite repressor 1 increases cellulase production in *Trichoderma reesei*. *Biotechnology for Biofuels*, 7(1), 129. Available from: https://doi.org/10.1186/s13068-014-0129-3

Meyer, V., Arentshorst, M., El-Ghezal, A., Drews, A.-C., Kooistra, R., van den Hondel, C.A.M.J.J. et al. (2007) Highly efficient gene targeting in the *Aspergillus niger* kusA mutant. *Journal of Biotechnology*, 128(4), 770–775. Available from: https://doi.org/10.1016/j.jbiotec.2006.12.021

Meyer, V., Ram, A.F.J. & Punt, P.J. (2010) Genetics, genetic manipulation, and approaches to strain improvement of filamentous fungi. In: Baltz, R.H., Davies, J.E., Demain, A.L., Bull, A.T., Junker, B. & Katz, L. (Eds.) *Manual of industrial microbiology and biotechnology*, 3rd edition. Washington, DC, USA: John Wiley & Sons, Inc, pp. 318–329.

Meyer, V., Wanka, F., van Gent, J., Arentshorst, M., van den Hondel, C.A.M.J.J. & Ram, A.F.J. (2011) Fungal gene expression on demand: an inducible, tunable, and metabolism-independent expression system for *Aspergillus niger*. *Applied and Environmental Microbiology*, 77(9), 2975–2983. Available from: https://doi.org/10.1128/AEM.02740-10

Nakari-Setälä, T., Paloheimo, M., Kallio, J., Vehmaanperä, J., Penttilä, M. & Saloheimo, M. (2009) Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. *Applied and Environmental Microbiology*, 75(14), 4853–4860. Available from: https://doi.org/10.1128/AEM.00282-09

Nehlin, J.O. & Ronne, H. (1990) Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *The EMBO Journal*, 9(9), 2891–2898. Available from: https://doi.org/10.1002/j.1460-2075.1990.tb07479.x

Niu, J., Arentshorst, M., Seelinger, F., Ram, A.F.J. & Ouedraogo, J.P. (2016) A set of isogenic auxotrophic strains for constructing multiple gene deletion mutants and parasexual crossings in *Aspergillus niger*. *Archives of Microbiology*, 198(9), 861–868. Available from: https://doi.org/10.1007/s00203-016-1240-6

Peng, M., Khosravi, C., Lubbers, R.J.M., Kun, R.S., Pontes, A., Battaglia, E. et al. (2021) CreA-mediated repression of gene expression occurs at low monosaccharide levels during fungal plant biomass conversion in a time and substrate dependent manner. *Cell Surface (Amsterdam, Netherlands)*, 7, 100050. Available from: https://doi.org/10.1016/j.tcsw.2021.100050

Prathumpai, W., McIntyre, M. & Nielsen, J. (2004) The effect of CreA in glucose and xylose catabolism in *Aspergillus nidulans*. *Applied Microbiology and Biotechnology*, 63(6), 748–753. Available from: https://doi.org/10.1007/s00253-003-1409-1

Rassinger, A., Gacek-Matthews, A., Strauss, J., Mach, R.L. & Mach-Aigner, A.R. (2018) Truncation of the transcriptional repressor protein Cre1 in *Trichoderma reesei* rut-C30 turns it into an activator. *Fungal Biology and Biotechnology*, 5, 15. Available from: https://doi.org/10.1186/s40694-018-0059-0

Revilla, G., López-Nieto, M.J., Luengo, J.M. & Martín, J.F. (1984) Carbon catabolite repression of penicillin biosynthesis by *Penicillium chrysogenum*. *The Journal of Antibiotics*, 37(7), 781–789. Available from: https://doi.org/10.7164/antibiotics.37.781

Ries, L.N.A., Beattie, S.R., Espeso, E.A., Cramer, R.A. & Goldman, G.H. (2016) Diverse regulation of the CreA carbon catabolite repressor in *Aspergillus nidulans*. *Genetics*, 203(1), 335–352. Available from: https://doi.org/10.1534/genetics.116.187872

Ries, L.N., Alves de Castro, P., Pereira Silva, L., Valero, C., Dos Reis, T.F., Saborano, R. et al. (2021) Aspergillus fumigatus acetate utilization impacts virulence traits and pathogenicity. *MBio*, 12(4), e0168221. Available from: https://doi.org/10.1128/mBio.01682-21

Robl, D., Benoit, I., Padilla, G., Pradella, D.C., Geraldo, J. & Vries, R.P. (2018) Improved hemicellulase production by genetic modification of carbon catabolite repression and xylanolitic activation in *Aspergillus niger*. *Current Biotechnology*, 7, 10–18.

Roy, P., Lockington, R.A. & Kelly, J.M. (2008) CreA-mediated repression in *Aspergillus nidulans* does not require transcriptional auto-regulation, regulated intracellular localisation or degradation of CreA. *Fungal Genetics and Biology*, 45(5), 657–670. Available from: https://doi.org/10.1016/j.fgb.2007.10.016

Ruijter, G.J. & Visser, J. (1997) Carbon repression in aspergilli. *FEMS Microbiology Letters*, 151(2), 103–114. Available from: https://doi.org/10.1111/j.1574-6968.1997.tb12557.x

Ruijter, G.J.G., Vanhanen, S.A., Gielkens, M.M.C., van de Vondervoort, P.J.I. & Visser, J. (1997) Isolation of *Aspergillus niger* creA mutants and effects of the mutations on expression of arabinases and L-arabinose catabolic enzymes. *Microbiology (Reading, England)*, 143(Pt 9), 2991–2998. Available from: https://doi.org/10.1099/00221287-143-9-2991

Sambrook, J. & Russell, D.W. (2001) *Molecular cloning: a laboratory manual*, Vol. 1, 3rd edition. New York: Cold Spring Harbor Laboratory Press.

Sauer, M., Porro, D., Mattanovich, D. & Branduardi, P. (2008) Microbial production of organic acids: expanding the markets. *Trends in Biotechnology*, 26(2), 100–108. Available from: https://doi.org/10.1016/j.tibtech.2007.11.006

Schäfer, D., Schmitz, K., Weuster-Botz, D. & Benz, J.P. (2019) Comparative evaluation of *Aspergillus niger* strains for endogenous pectin depolymerization capacity and suitability for D-galacturonic acid production. *Bioprocess and Biosystems Engineering*, 43(9), 1549–1560.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T. et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676–682. Available from: https://doi.org/10.1038/nmeth.2019

Schmitz, K., Protzko, R., Zhang, L. & Benz, J.P. (2019) Spotlight on fungal pectin utilization-from phytopathogenicity to molecular recognition and industrial applications. *Applied Microbiology and Biotechnology*, 103(6), 2507–2524. Available from: https://doi.org/10.1007/s00253-019-09622-4

Shinmyo, A., Davis, I.K., Nomoto, F., Tahara, T. & Enatsu, T. (1978) Catabolite repression of hydrolases in *Aspergillus niger*. *European Journal of Applied Microbiology and Biotechnology*, 5(1), 59–68. Available from: https://doi.org/10.1007/BF00515687

Shroff, R.A., Lockington, R.A. & Kelly, J.M. (1996) Analysis of mutations in the creA gene involved in carbon catabolite repression in *Aspergillus nidulans*. *Canadian Journal of Microbiology*, 42(9), 950–959. Available from: https://doi.org/10.1139/m96-122

Shroff, R.A., O'Connor, S.M., Hynes, M.J., Lockington, R.A. & Kelly, J.M. (1997) Null alleles of creA, the regulator of carbon catabolite repression in *Aspergillus nidulans*. *Fungal Genetics and Biology*, 22(1), 28–38. Available from: https://doi.org/10.1006/fgbi.1997.0989

Song, D., Shi, Y., Ji, H.Q., Xia, Y. & Peng, G. (2019) The MaCreA gene regulates normal conidiation and microcycle conidiation in *Metarhizium acridum*. *Frontiers in Microbiology*, 10, 1946. Available from: https://doi.org/10.3389/fmicb.2019.01946

Strauss, J., Mach, R.L., Zeilinger, S., Hartler, G., Stöffler, G., Wolschek, M. et al. (1995) Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. *FEBS Letters*, 376(1–2), 103–107. Available from: https://doi.org/10.1016/0014-5793(95)01255-5

Sun, J. & Glass, N.L. (2011) Identification of the CRE-1 cellulolytic regulon in *Neurospora crassa*. *PLoS One*, 6(9), e25654. Available from: https://doi.org/10.1371/journal.pone.0025654

Sun, J., Tian, C., Diamond, S. & Glass, N.L. (2012) Deciphering transcriptional regulatory mechanisms associated with hemicellulose degradation in *Neurospora crassa*. *Eukaryotic Cell*, 11(4), 482–493. Available from: https://doi.org/10.1128/EC.05327-11

Tamayo, E.N., Villanueva, A., Hasper, A.A., Graaff, L.H., Ramón, D. & Orejas, M. (2008) CreA mediates repression of the regulatory gene xlnR which controls the production of xylanolytic enzymes in *Aspergillus nidulans*. *Fungal Genetics and Biology*, 45(6), 984–993. Available from: https://doi.org/10.1016/j.fgb.2008.03.002

Tamayo-Ramos, J.A., Flipphi, M., Pardo, E., Manzanares, P. & Orejas, M. (2012) L-rhamnose induction of *Aspergillus nidulans* α-L-rhamnosidase genes is glucose repressed via a CreA-independent mechanism acting at the level of inducer uptake.

Microbial Cell Factories, 11, 26. Available from: https://doi.org/10.1186/1475-2859-11-26

Tanco, S., Gevaert, K. & van Damme, P. (2015) C-terminomics: targeted analysis of natural and posttranslationally modified protein and peptide C-termini. *Proteomics*, 15(5–6), 903–914. Available from: https://doi.org/10.1002/pmic.201400301

Timberlake, W.E. & Marshall, M.A. (1989) Genetic engineering of filamentous fungi. *Science*, 244(4910), 1313–1317. Available from: https://doi.org/10.1126/science.2525275

van Gooch, D., Mehra, A., Larrondo, L.F., Fox, J., Touroutoutoudis, M., Loros, J.J. et al. (2008) Fully codon-optimized luciferase uncovers novel temperature characteristics of the Neurospora clock. *Eukaryotic Cell*, 7(1), 28–37. Available from: https://doi.org/10.1128/EC.00257-07

Vautard-Mey, G. & Fèvre, M. (2000) Mutation of a putative AMPK phosphorylation site abolishes the repressor activity but not the nuclear targeting of the fungal glucose regulator CRE1. *Current Genetics*, 37(5), 328–332. Available from: https://doi.org/10.1007/s002940050535

Vogel, H.J. (1956) A convenient growth medium for *Neurospora crassa*. *Genetic Bulletin*, 61(3), 643.

Westholm, J., Nordberg, N., Murén, E., Ameur, A., Komorowski, J. & Ronne, H. (2008) Combinatorial control of gene expression by the three yeast repressors Mig1, Mig2 and Mig3. *BMC Genomics*, 9, 601. Available from: https://doi.org/10.1186/1471-2164-9-601

Wu, V.W., Thieme, N., Huberman, L.B., Dietschmann, A., Kowbel, D.J., Lee, J. et al. (2020) The regulatory and transcriptional landscape associated with carbon utilization in a filamentous fungus. *Proceedings of the National Academy of Sciences of the United States of America*, 117(11), 6003–6013. Available from: https://doi.org/10.1073/pnas.1915611117

Zhang, J., Wu, C., Wang, W. & Wei, D. (2018) Construction of enhanced transcriptional activators for improving cellulase production in *Trichoderma reesei* RUT C30. *Bioresources and Bioprocessing*, 5(1), 40. Available from: https://doi.org/10.1186/s40643-018-0226-4

Zhang, J., Zhang, G., Wang, W. & Wei, D. (2018) Enhanced cellulase production in *Trichoderma reesei* RUT C30 via constitution of minimal transcriptional activators. *Microbial Cell Factories*, 17(1), 75. Available from: https://doi.org/10.1186/s12934-018-0926-7

Zhang, X., Zhu, Y., Bao, L., Gao, L., Yao, G., Li, Y. et al. (2016) Putative methyltransferase LaeA and transcription factor CreA are necessary for proper asexual development and controlling secondary metabolic gene cluster expression. *Fungal Genetics and Biology*, 94, 32–46. Available from: https://doi.org/10.1016/j.fgb.2016.07.004

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