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D-Xylose Concentration-Dependent Hydrolase Expression Profiles and the Function of CreA and XlnR in *Aspergillus niger*

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***Aspergillus niger* is an important organism for the production of industrial enzymes such as hemicellulases and pectinases. The xylan-backbone monomer, D-xylose, is an inducing substance for the coordinate expression of a large number of polysaccharide-degrading enzymes. In this study, the responses of 22 genes to low (1 mM) and high (50 mM) D-xylose concentrations were investigated. These 22 genes encode enzymes that function as xylan backbone-degrading enzymes, accessory enzymes, cellulose-degrading enzymes, or enzymes involved in the pentose catabolic pathway in *A. niger*. Notably, genes encoding enzymes that have a similar function (e.g., xylan backbone degradation) respond in a similar manner to different concentrations of D-xylose. Although low D-xylose concentrations provoke the greatest change in transcript levels, in particular, for hemicellulase-encoding genes, transcript formation in the presence of high concentrations of D-xylose was also observed. Interestingly, a high D-xylose concentration is favorable for certain groups of genes. Furthermore, the repressing influence of CreA on the transcription and transcript levels of a subset of these genes was observed regardless of whether a low or high concentration of D-xylose was used. Interestingly, the decrease in transcript levels of certain genes on high D-xylose concentrations is not reflected by the transcript level of their activator, XlnR. Regardless of the D-xylose concentration applied and whether CreA was functional, *xlnR* was constitutively expressed at a low level.**

Aspergillus niger is a filamentous ascomycete that degrades plant biomass wherever available due to its saprophytic activity. Therefore, this fungus produces a broad spectrum of hydrolytic enzymes; examples of enzymes that degrade the xylan backbone are endoxylanases and a β-xylosidase as well as the accessory enzymes that remove the modifications of the backbone residues. The sugars that are released after degradation are mostly pentoses such as D-xylose and L-arabinose that are metabolized intracellularly. Table 1 summarizes the *A. niger* enzymes and their encoding genes that are involved in the degradation of xylan and metabolism of the D-xylose that is released.

Some of these enzymes are produced in industrially relevant amounts; *A. niger* is used as a native production host for enzymes such as pectinases (4, 8, 31) and hemicellulases like xylanases or arabinases (20, 46). Consequently, the induction of hemicellulase expression using inexpensive substances is an important issue. In the past, the xylan backbone monosaccharide, D-xylose, was shown to trigger expression of xylanases and D-xylose-metabolizing enzymes in *A. niger*. Therefore, D-xylose is commonly used to induce the expression of these enzymes (1, 7, 13, 43). Moreover, the coregulation of the xylanolytic and the cellulolytic systems via the inducer D-xylose has been reported (12, 19, 22).

The *A. niger* protein XlnR is a binuclear zinc finger protein (48) that belongs to a class of transcription factors specific for fungi, such as the *Saccharomyces cerevisiae* protein GAL4p (17). XlnR functions as a transactivator responsible for a wide range of target genes such as those encoding xylan-degrading enzymes or the enzymes involved in D-xylose metabolism (7, 13, 22, 47), those encoding endocellulases and cellobiohydrolases (19), and some of the galactosidase-encoding genes (11). Within the pool of possible XlnR target genes, only the *bglA* gene was clearly shown not to be under the control of XlnR (19); there are contradicting reports for the *xkiA* gene (7, 44). Whereas XlnR functions as the transactivator of polysaccharide-degrading enzyme expression, CreA has been described as a wide-domain regulator mediating carbon catabolite repression and is best studied in *Aspergillus nidulans* (reviewed in references 2, 37, and 39). In *A. niger*, repression of xylanase gene expression in the presence of glucose was correlated to CreA influence (5, 15). In 1999, de Vries et al. reported that CreA also mediates carbon catabolite repression of genes involved in xylan degradation on D-xylose at concentrations higher than 1 mM (14).

In this study, we investigated how genes encoding arabinoxylan-degrading enzymes that are classified by their particular activity (xylan backbone-degrading enzymes, accessory enzymes, cellulose-degrading enzymes, and D-xylose metabolizing enzymes) respond to high and low D-xylose concentrations. We also investigated whether the response of enzymes within the group is homogenous and whether the response of different groups of enzymes changes over time.

Furthermore, we addressed the question of whether the carbon catabolite-repressing signal of D-xylose is mediated by CreA and whether this occurs via a concentration-dependent mechanism. Finally, we examined whether a D-xylose concentration-dependent effect on the transcript levels of target genes is reflected by the transcript level of their main activator encoding gene, *xlnR*, and

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TABLE 1 *A. niger* enzymes involved in the degradation of xylan

Enzyme	EC no.	GH family no.	Gene	GenBank accession no.	Reference
Xylan backbone-modifying enzymes					
Endoxylanase B	EC 3.2.1.8	11	*xlnB*	D38071	24
Endoxylanase C	EC 3.2.1.8	10	*xlnC*	C5J411	M. M. C. Gielkens, J. Visser, and L. H. de Graaff, unpublished data
β-Xylosidase	EC 3.2.1.37	3	*xlnD*	Z84377	46
Xylan accessory enzymes					
α-L-Arabinofuranosidase B	EC 3.2.1.55	54	*abfB*	L23502	18
α-Galactosidase B	EC 3.2.1.22	27	*aglB*	Y18586	11
α-Glucuronidase	EC 3.2.1.139	67	*aguA*	AJ290451	10
Acetyl xylan esterase	EC 3.2.1.72		*axeA*	A22880	6
Arabinoxylan arabinofuranohydrolase	EC 3.2.1.55	62	*axhA*	Z78011	20
Esterase A			*estA*	AY456379	
Ferulic acid esterase	EC 3.1.1.73		*faeA*	Y09330	9
β-Galactosidase	EC 3.2.1.23	35	*lacA*	L06037	11
Cellulose-degrading enzymes					
β-Glucosidase	EC 3.2.1.21	3	*bglA*	AF121777	47
Cellobiohydrolase A	EC 3.2.1.91	7	*cbhA*	AF156268	19
Cellobiohydrolase B	EC 3.2.1.91	7	*cbhB*	AF156269	19
Endoglucanase A	EC 3.2.1.4	12	*eglA*	AJ224451	47
Endoglucanase B	EC 3.2.1.4	5	*eglB*	AJ224452	47
Endoglucanase C	EC 3.2.1.4	74	*eglC*	AY040839	
D-Xylose metabolism enzymes					
L-Arabitol dehydrogenase	EC 1.1.1.12		*ladA*	AJ854040	38
Transaldolase B	EC 2.2.1.2		*talB*	XM001391362	
Xylitol dehydrogenase	EC 1.1.1.9		*xdhA*	AJ854041	44
D-Xylulose kinase	EC 2.7.1.17		*xkiA*	AJ305311	3
Xylose reductase	EC 1.1.1.21		*xyrA*	AF219625	22

whether regulation of *xlnR* expression by CreA might be responsible for such an effect.

MATERIALS AND METHODS

Strains and growth conditions. The *A. niger* strains N400 (CBS 120.49) and NW283 (*cspA1 fwnA1 pyrA6 lysA7 creA^{d}4*) were maintained for generating spores on complete medium plates (34) supplemented with uridine and lysine. Strain NW283 exhibits a CreA-derepressed phenotype (creA^{d}4 [d, derepressed; 4, allele 4]) and was previously used for studying CreA effects in *A. niger* (14, 21, 35, 44, 45). Strain NW283 is a derivative of the NW145 strain (bearing additionally a *pyrA* marker) and is therefore a descendant of the N400 strain (36), to which it was consequently compared.

Cultivation of the strains in 2.2-liter benchtop fermentors (Applikon Biotechnology, Schiedam, The Netherlands) was carried out in duplicate using 2 liters of medium at pH 6.0 supplemented with uridine and lysine and containing 1.2 g $NaNO_3$, 0.3 g KH_2PO_4, 0.1 g KCl, 0.1 g $MgSO_4 \cdot 7 H_2O$, 2 g yeast extract, 4 g Casamino Acids, and 2 ml Vishniac solution (49). The carbon source was 100 mM sorbitol. To avoid excessive foam formation, 1 ml antifoam (Sigma-Aldrich, St. Louis, MO) was added to the medium. Inoculation of the cultures using 1×10^6 spores per ml, cultivation conditions, and induction with 1 mM or 50 mM D-xylose were as previously published (43). After induction, the reference sample was taken within 30 s (preinduction sample, identical to the 0-h sample). After induction, samples were collected each hour (10 ml each, postinduction samples) and analyzed by microscopy for infection control. Culture supernatant and mycelia were separated by filtration through Miracloth (Calbiochem [part of Merck, Darmstadt], Germany). Mycelia were immediately snap-frozen in liquid nitrogen.

TABLE 2 Primers used for qPCR in this study

Gene	Primer sequence (5′→3′)	
	Forward	Reverse
abfB	GATAGTTCCACCACGCTGAAGG	CTGCTTCGTGCCATCGTTGG
aglB	GATATCACGGCAACCTGGTCC	CTTCATCATCGCCCAAAGGG
aguA	CACAGTTCGGATCTTAGGCAAGG	GTCCATGTTGTCCCATTGATTCACC
axeA	CTATGATGACGAACGTAATGGC AGC	GACGTCTCCTTGTGCGCAG
axhA	CTCCAGCACTTTCACCTACCG	CACAGTCTGATCAATGGCACCG
bglA	GACCGCCAGTGTCTCTCTTG	GATCGTGTTGTTGCAGTTGCTAGC
cbhA	CACTGGCAACACCTGGGATG	GTCAGTGGTGACACCGTAGG
cbhB	CAATGTGCGACGGTGACTCC	GTGATGAACTGGGTGACGACG
eglA	GTGGTGAGGGAACAGTGAAAAGC	GACATCGGCGTTGACGTTGG
eglB	GACTGGTTCATACGACGAGGAG	GTATCAAACATGACCAAGTCGTTATC
eglC	ACGGACTACGGCGAGACATG	CACCACACCGACAAAGATGCG
estA	CAAAGTGCCACGGAGTACGG	GTAGTTTCGATTGAGATGTGGCTGTGG
faeA	ACCGTGACAGGCCATAGTCTG	GGAAGTACTGGGTCGTCTCC
hist	ATCTTGCGTGACAACATCCA	CACCCTCAAGGAAGGTCTTG
lacA	CTGGATGCTACAGATAATTACG TCTCTC	CATACTGCATGTACACTGGATCGG
ladA	GAATCTGCGGGTCCGACGTG	GCAAATGATGTTAGGCTCGACGG
spas	ACTCCAGAGGACAAGCAGGA	GCAGACGCATGCTCTCAATA
talB	GCACCACAGTCGCCAAAACC	GGTATTCTTGGATGAGGGCTTTGG
xdhA	CAGGTATATGTGGTAGTGATGTT CATTATTGG	CATTGCAACGCAGTCTCCTACC
xkiA	AGCAAGGCACACCATAGATT TACAGG	CATCAGAGATGTCCATCGGAGC
xlnB	CAACTTTGTCGGTGGAAAGG	GGGTAGCCGTGTAGATATCG
xlnC	GTGGAGGTGCTGGAATTTCTGG	CAGGAATCCGGGTCAGCAAC
xlnD	TAATCTACGCCGGTGGTATC	TTCTTGAGCGAAGAGGAATC
xlnR	CTCATTGCATTGAATTCGGA CTGACC	GCTGTCTTCCGACGTTCGC
xyrA	ACGGCGCCTGCGACTATG	TGCGGCAAATGGGCTCGAC

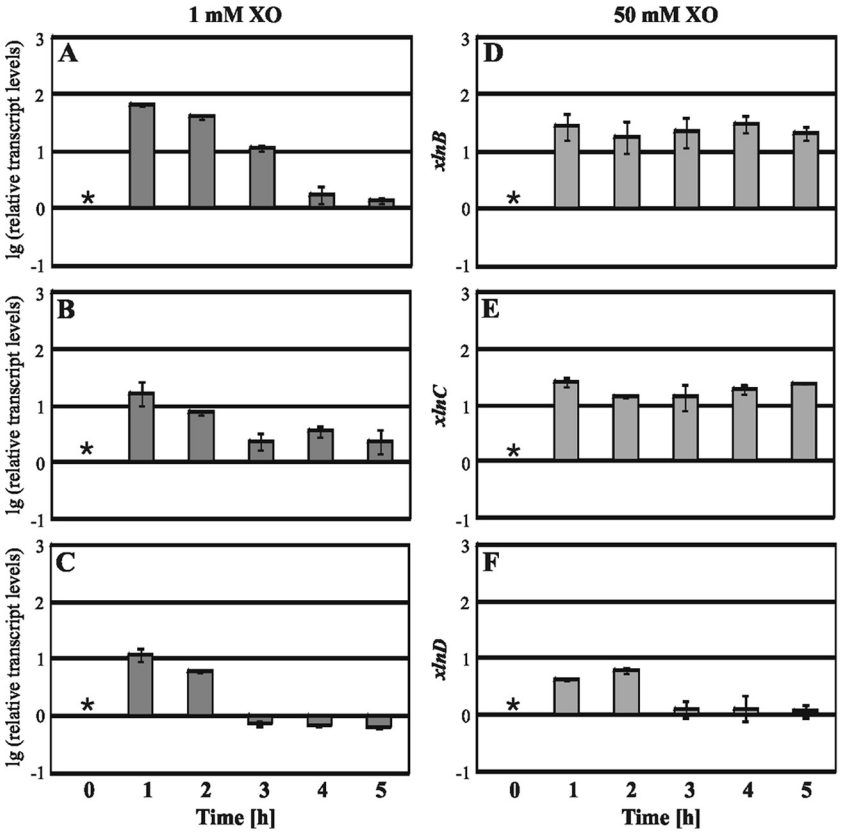


FIG 1 Transcript levels of genes encoding xylan backbone-degrading enzymes in *A. niger*. Strain N400 was induced using 1 or 50 mM D-xylose (XO), and samples were taken hourly for 5 h. Transcript level analysis of *xlnB* (A and D), *xlnC* (B and E), and *xlnD* (C and F) was performed by qPCR using a histone-like gene transcript and a *Schizosaccharomyces pombe dma1*-like gene transcript for normalization. Transcript levels always refer to the reference sample (taken directly after induction) within an experiment, which is indicated by an asterisk. The results are presented as the relative transcript levels in logarithmic scale (lg). The values are means from two independent biological experiments measured in triplicate. Error bars indicate the standard deviations.

RNA extraction and reverse transcription (RT). Harvested mycelia were homogenized in 1 ml of peqGOLD TriFast DNA/RNA/protein purification system reagent (Peqlab Biotechnologie, Erlangen, Germany) using a FastPrep FP120 BIO101 ThermoSavant cell disrupter (Qbiogene, Carlsbad, CA). RNA was isolated according to the manufacturer's instructions, and the concentration was measured using a NanoDrop 1000 (Thermo Scientific, Waltham, MA).

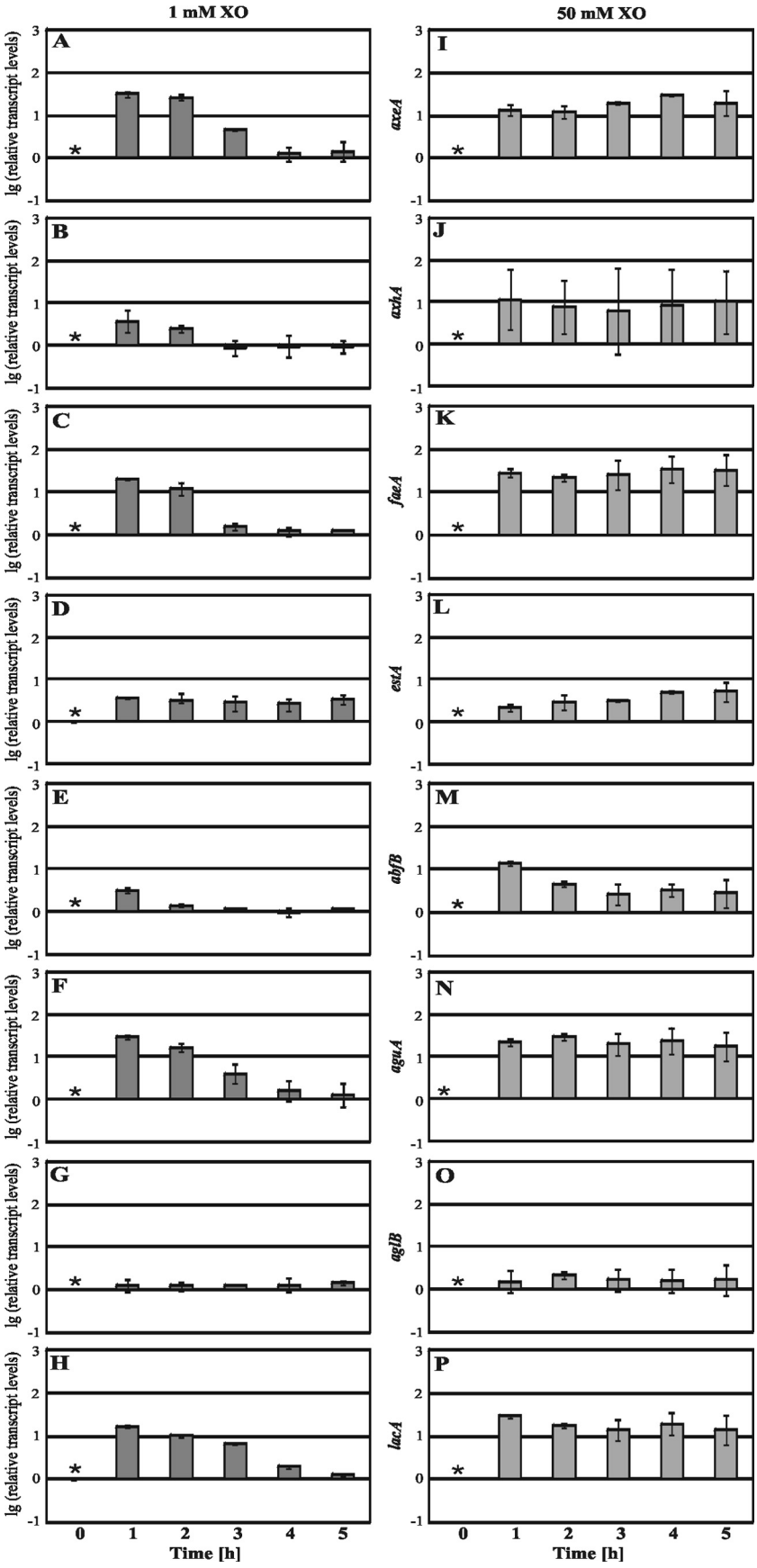
After treatment with DNase I (Fermentas [part of Thermo Fisher Scientific], St. Leon-Rot, Germany), cDNA was synthesized from 0.45 μg mRNA using the RevertAid H Minus first-strand cDNA synthesis kit (Fermentas,); all reactions were performed according to the manufacturer's instructions.

qPCR analysis. All quantitative PCRs (qPCRs) were performed in triplicate in a Rotor-Gene Q cycler (Qiagen, Hilden, Germany). The amplification mixture (final volume, 15 μl) contained 7.5 μl 2× ABsolute QPCR SYBR Green mix (ABgene [part of Thermo Fisher Scientific], Cambridge, United Kingdom), 100 nM forward and reverse primers and 2.5 μl cDNA (diluted 1:100). Primer sequences are provided in Table 2. Each experiment included a template-free control and an amplification-inhibited control (with 0.015% SDS added to the reaction mixture). The cycling conditions comprised a 15-min initial polymerase activation at 95°C, followed by 40 cycles of 95°C for 15 s, 59°C for 15 s, and 72°C for 15 s. Initially, four previously used reference genes (43) were compared to evaluate how stable their expression is under the applied conditions. Therefore, a synthetic control RNA transcript, a bacterial kanamycin synthetase-encoding gene fused to a eukaryotic poly(A) tail (Promega), was added to the total RNA prior to cDNA synthesis to correct for various efficiencies of the reverse transcription or PCR itself (23). The differences in the expression of all four candidate reference genes were below 5% under all conditions. Consequently, the number of the genes used for normalization of the

TABLE 3 HPLC analysis of D-xylose and sorbitol concentrations in supernatants of *A. niger* N400 cultivations in benchtop fermentors

Target D-xylose concn (mM)	Time (h) after induction	Concn (mM)[a]	
		D-Xylose	Sorbitol
1	0	0.7 ± 0.2	85.4 ± 2.3
	1	0.6 ± 0.1	77.2 ± 1.0
	2	ND[b]	70.9 ± 1.0
	3	ND	65.7 ± 2.7
	4	ND	56.1 ± 3.5
	5	ND	48.0 ± 3.3
50	0	55.1 ± 0.8	91.2 ± 3.7
	1	52.8 ± 0.9	85.2 ± 2.5
	2	52.1 ± 0.5	83.4 ± 4.6
	3	47.9 ± 1.7	78.7 ± 8.3
	4	45.5 ± 0.2	75.3 ± 5.8
	5	43.1 ± 0.1	70.1 ± 5.3

[a] Values are means and standard deviations from two biological experiments.
[b] ND, not detected; the limit of quantification was <0.03 mM.



expression data was reduced to two, which were randomly chosen: the histone-encoding gene (termed "*hist*") and the gene with similarity to the *Schizosaccharomyces pombe* gene encoding Dma1, which is a component of the spindle assembly point involved in mitotic division (termed "*spas*"). Calculations were performed using the equation from Pfaffl (33): the transcript ratio equals $E_{\text{target gene}}\wedge[\Delta C_T(\text{pre} - \text{post})_{\text{target gene}}]/E_{\text{reference gene}}\wedge[\Delta C_T(\text{pre} - \text{post})_{\text{reference gene}}]$, where E denotes the amplification efficiency, and $\Delta C_T(\text{pre} - \text{post})$ denotes the difference in threshold cycles between pre- and postinduction samples.

Statistical analyses of qPCR data. The log-transformed data values of the relative transcript level ratios were used for the comparative data analysis. Sample standard deviations were computed for every data point for the entire data set using the averaged log-relative transcript level ratios from the duplicate experiments. The expression for computing the unbiased sample standard deviation (SD) is given by

$$\text{SD}[\log(y_{ijd})] = \sqrt{\frac{1}{m-1}\sum_{d=1}^{m}[\log(y_{ijd}) - \overline{\log(y_{ijd})}]^2}$$

where $\log(y_{ijd})$ and $\overline{\log(y_{ijd})}$ are the log-relative transcript level and mean log-relative transcript level ratio, respectively. The indices *i*, *j*, and *d* represent the profiled genes, the time instant, and the duplicates, respectively. In this case, $j = 1, \ldots, N (= 6)$ corresponds to the zero- to fifth-hour mark (where N is the total number of samples taken in time) and $d = 1, \ldots, m (= 2)$. Overlapping standard error bars indicate no significant difference in transcript levels between groups. This approach provides a standard platform for comparing the transcript levels of the genes as a result of induction by 1 mM and 50 mM D-xylose. A similar analysis was used to compare gene transcript levels between the wild-type and CreA mutant strains.

HPLC analysis. Analyses were performed using a Thermo Finnigan Surveyor high-pressure liquid chromatography (HPLC) instrument (Thermo Fisher Scientific, MA). All 10-μl samples were injected onto a Repro-Gel Pb column (9 μm, 150 by 8 mm; Dr. Maisch GmbH, Germany). Water was used as the mobile phase, and elution was followed at 50°C at a flow rate of 1.0 ml/min for 20 min. The concentration was determined using xylitol as an internal standard.

RESULTS

Response of xylan backbone-degrading enzyme expression to different D-xylose concentrations. *A. niger* strain N400 was cultivated in a benchtop fermentor, and a low (1 mM final concentration) or high (50 mM final concentration) concentration of D-xylose was added. Samples were taken immediately after the pulse and then every 60 min after the initial sample to compare transcript formation. The genes encoding endoxylanase B (*xlnB*) and endoxylanase C (*xlnC*) as well as β-xylosidase (*xlnD*) were analyzed by RT-qPCR. The use of 1 mM D-xylose leads to high transcript levels for all three genes after 1 h, with a slight decrease in the transcript levels of these genes after 2 h (Fig. 1A to C). HPLC analysis showed that D-xylose was not detectable after 2 h (Table 3). Interestingly, after 3 h, the transcript formation trend clearly differs: while considerable amounts of the *xlnB* transcript could be detected, which decreased at later a time point (Fig. 1A), *xlnC* transcript levels dropped significantly and then remained at a low constant level (Fig. 1B), and *xlnD* transcript formation was reduced below the preinduction level (Fig. 1C). These differences might be due to differences in the mRNA stability of each transcript. In contrast to the case for 1 mM D-xylose, the addition of 50 mM D-xylose to the culture induces *xlnB* and *xlnC* at a constant level over the entire period investigated (Fig. 1D and E). However, transcription of *xlnD* is induced for only 2 h and then decreases to nearly preinduction levels (Fig. 1F). Because there are other genes known to be induced by D-xylose, we also investigated their response to different concentrations of D-xylose.

Expression of accessory enzyme-encoding genes is mostly favored using a high D-xylose concentration. We used the samples from *A. niger* N400 mycelia generated as described in Materials and Methods to analyze the expression of different genes encoding accessory enzymes, namely, *axeA*, *axhA*, *faeA*, *estA*, *abfB*, *aguA*, *aglB*, and *lacA*. Overall, we found that the change in transcript level of *axeA* at 1 and 2 h after induction as well as the levels of *estA* and *aguA* after 1 h are higher in the samples induced with 1 mM D-xylose (Fig. 2A, D, and F) than in those induced with 50 mM D-xylose (Fig. 2I, L, and N). For all the other genes, in particular, at the later sampling time points (3, 4, and 5 h after the addition of D-xylose), we detected higher transcript levels in the 50 mM D-xylose culture (compare Fig. 2A to H and I to P). This finding again correlates with the depletion of D-xylose in the culture after 2 h when the initial concentration was 1 mM (Table 3). Exceptions to this trend were *estA* and *aglB*, because they maintained constant transcript levels even after 3 h (Fig. 2D and G). However, *aglB* is not generally significantly induced. Overall, in particular, *axeA* and *aguA* respond to different D-xylose concentrations in the same way: the greatest change in transcript levels occurs with 1 mM D-xylose after 1 or 2 h, but a constant, highly induced level over time was obtained with 50 mM D-xylose. However, all genes show a prolonged induction in the presence of the high D-xylose concentration.

Potential of D-xylose to induce expression of cellulase-encoding genes. Because it was reported that the xylanolytic and cellulolytic enzyme systems are coordinately expressed in *A. niger* (reviewed in references 12 and 41), we investigated the response of genes encoding cellulose-degrading enzymes to low and high D-xylose concentrations. Using 1 mM D-xylose triggered the induction of *eglA* and *bglA* (Fig. 3C and F) and low induction of *cbhA* and *eglB* (Fig. 3A and D), and the expression of *cbhB* and *eglC* was similar to that in the preinduction sample (Fig. 3B and E). While *cbhB* remained at a very low transcript level after addition of 50 mM D-xylose (Fig. 3H), *eglC* transcript formation increased significantly (Fig. 3K). Compared to the low D-xylose concentration, the higher D-xylose concentration resulted in increased transcript levels of *cbhA* (Fig. 3G) and *eglA* (Fig. 3I), while *eglB* (Fig. 3J) and *bglA* (Fig. 3L) exhibited reduced expression. Altogether, we found that D-xylose is not a very good inducer of cellulases, except for *eglA*, *bglA*, and, at higher concentrations, *eglC*. The use of a higher D-xylose concentration appears to be beneficial primarily for *eglC* transcript expression.

FIG 2 Transcript levels of genes encoding other accessory enzymes in *A. niger*. Strain N400 was induced using 1 or 50 mM D-xylose (XO), and samples were taken hourly for 5 h. Transcript level analysis of *axeA* (A and I), *axhA* (B and J), *faeA* (C and K), *estA* (D and L), *abfB* (E and M), *aguA* (F and N), *aglB* (G and O), and *lacA* (H and P) was performed by qPCR using a histone-like gene transcript and a *Schizosaccharomyces pombe dma1*-like gene transcript for normalization. Transcript levels always refer to the reference sample (taken directly after induction) within an experiment, which is indicated by an asterisk. The results are presented as the relative transcript levels in logarithmic scale (lg). The values are means from two independent biological experiments measured in triplicate. Error bars indicate the standard deviations.

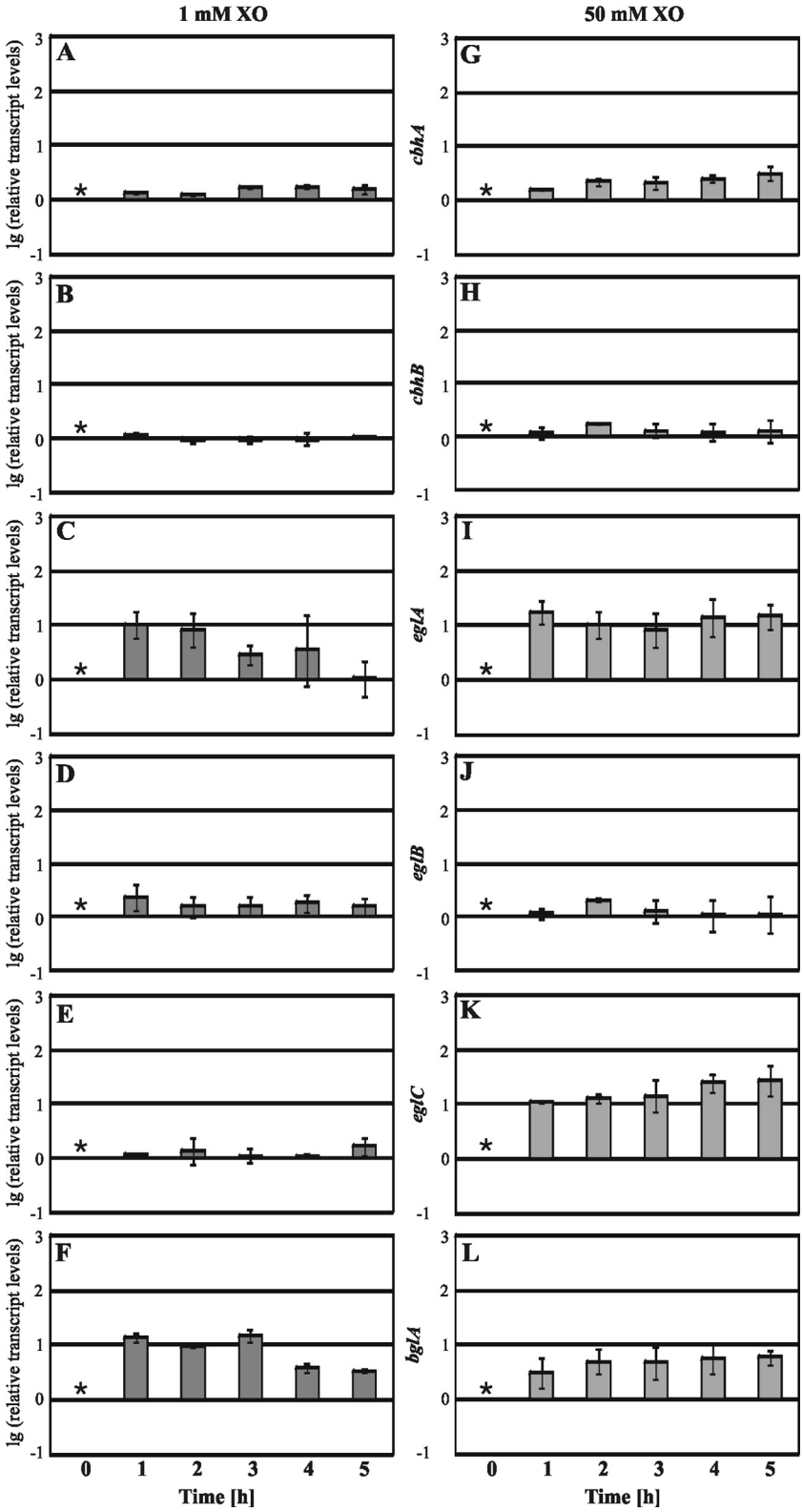


FIG 3 Transcript levels of genes encoding cellulose-degrading enzymes in *A. niger*. Strain N400 was induced using 1 or 50 mM D-xylose (XO), and samples were taken hourly for 5 h. Transcript level analysis of *cbhA* (A and G), *cbhB* (B and H), *eglA* (C and I), *eglB* (D and J), *eglC* (E and K), and *bglA* (F and L) was performed by qPCR using a histone-like gene transcript and a *Schizosaccharomyces pombe dma1*-like gene transcript for normalization. Transcript levels always refer to the reference sample (taken directly after induction) within an experiment, which is indicated by an asterisk. The results are presented as the relative transcript levels in logarithmic scale (lg). The values are means from two independent biological experiments measured in triplicate. Error bars indicate the standard deviations.

High D-xylose concentrations are preferable for inducing the gene expression of enzymes in the pentose metabolic pathway. According to microarray analysis data reported for *A. niger*, the expression of some genes coding for enzymes involved in the pentose metabolic pathway are upregulated on D-xylose compared to other carbon sources (1, 43). In this study, we investigated the influence of the concentration of D-xylose, which is one of the two starting metabolites of the pentose metabolic pathway, on regulation. We found that transcript formation of *xyrA*, *xdhA*, *ladA*, *xkiA*, and *talB* can be induced using 1 mM D-xylose for the first 2 h after induction and that after 2 h, expression strongly decreases (Fig. 4A to E). When 50 mM D-xylose is used for induction, the transcript formation of *xdhA*, *ladA*, and *xkiA* is even higher after 1 h than with 1 mM D-xylose. The observed transcript levels stay at this high level or at a slightly reduced level for the entire time period investigated (Fig. 4G to I). *xyrA*, which encodes the enzyme that directly metabolizes D-xylose (to xylitol), is an exception; after 1 h of induction, the change in transcript levels is significantly higher with 1 mM D-xylose than with 50 mM D-xylose (Fig. 4F). *talB* showed similar transcript levels after the first hour independent of the D-xylose concentration, but after 2 h, 50 mM D-xylose yielded enhanced transcript levels (Fig. 4J). Overall, the induction of the genes involved in pentose metabolism is stronger using a high D-xylose concentration, in particular, if observed for longer than 1 h.

Absence of a functional CreA positively influences expression of genes encoding xylan-degrading enzymes independent of D-xylose concentration. Previously, CreA-dependent decreased transcript levels of four xylanolytic enzyme-encoding genes in the presence of high D-xylose concentrations was observed by growth of *A. niger* with different D-xylose concentrations (14). To determine if and to what extent CreA influences the expression of genes encoding certain hemicellulases (i.e., *xlnB*, *xlnD*, *axeA*, *axhA*, *faeA*, and *aguA*) and *xyrA* on D-xylose, we grew the CreA mutant strain NW283 under the same conditions as the wild-type strain and analyzed gene expression using RT-qPCR. In the CreA mutant strain, the change in transcript levels of the observed genes generally increased (up to two orders of magnitude) relative to that in the wild-type strain, regardless of whether 1 mM or 50 mM D-xylose was used for induction (Fig. 5). The only exception was *xyrA*, which showed no significant difference in changes in transcript levels for the two strains (Fig. 5G and N). Interestingly, when comparing the transcript levels in the CreA mutant strain between samples derived from 1 mM D-xylose and those derived from 50 mM D-xylose, we found higher transcript levels after 1 and 2 h of induction when 1 mM D-xylose was used for induction (Fig. 5A to G) for all the genes investigated. Between 2 and 3 h, the D-xylose was depleted in the samples with the 1 mM initial D-xylose concentration (Table 4), which correlates with the distinct decrease in transcript levels after 3 h. In the presence of 50 mM D-xylose, slightly lower transcript levels were obtained but remained stable over time (Fig. 5H to N). In summary, a CreA deficiency leads to a strong enhancement of transcript formation that is independent of the concentration of D-xylose. This finding leads to the conclusion that CreA is involved in mediating the D-xylose repression signal via a concentration-independent mechanism.

The *xlnR* gene is constitutively expressed independent of CreA and D-xylose concentration. Because the repressor CreA is involved in regulation of hydrolase-encoding gene expression on

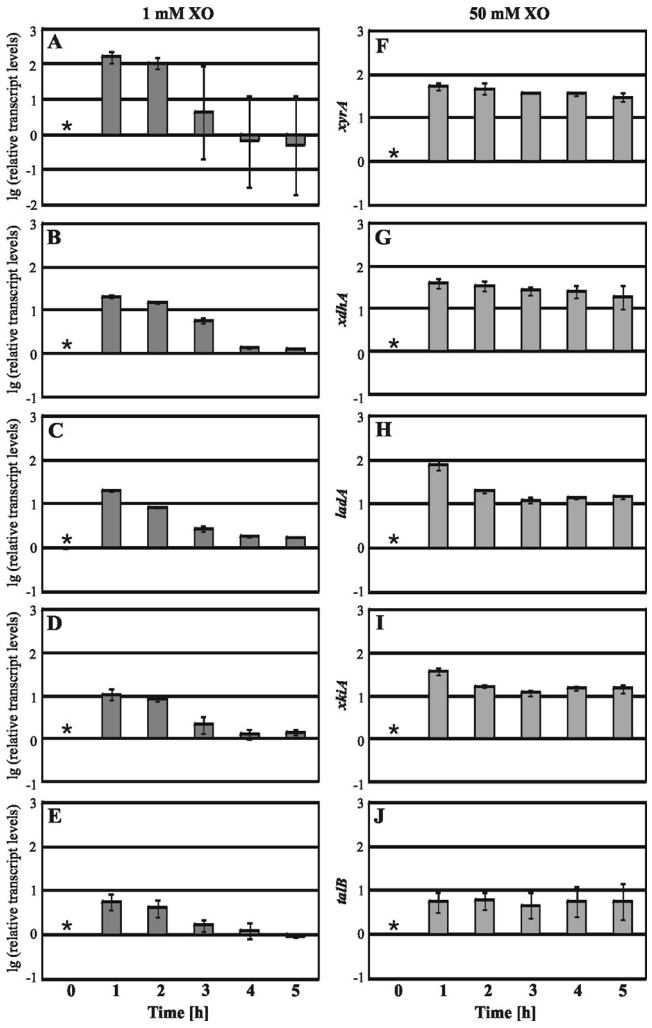


FIG 4 Transcript levels of genes encoding enzymes of the pentose metabolic pathway in *A. niger*. Strain N400 was induced using 1 or 50 mM D-xylose (XO), and samples were taken hourly for 5 h. Transcript level analysis of *xyrA* (A and F), *xdhA* (B and G), *ladA* (C and H), *xkiA* (D and I), and *talB* (E and J) was performed by qPCR using a histone-like gene transcript and a *Schizosaccharomyces pombe dma1*-like gene transcript for normalization. Transcript levels always refer to the reference sample (taken directly after induction) within an experiment, which is indicated by an asterisk. The results are presented as the relative transcript levels in logarithmic scale (lg). The values are means from two independent biological experiments measured in triplicate. Error bars indicate the standard deviations.

D-xylose, we asked if CreA might exert its effect indirectly by regulating the expression of the activator XlnR. Furthermore, it was unclear if the difference in the response to different D-xylose concentrations of certain genes, in particular, the ones encoding xylan backbone-degrading enzymes and some accessory enzymes, is provoked by a D-xylose concentration-dependent expression of their transcriptional activator. A comparison of *xlnR* transcript levels using 1 mM or 50 mM D-xylose shows similar constitutive transcript levels over the whole period investigated (Fig. 6A and B). Analysis of *xlnR* transcript levels in the CreA mutant also yielded no significant differences in expression, regardless of whether *xlnR* expression with different D-xylose concentrations for the CreA mutant strain was compared (Fig. 6C and D) or

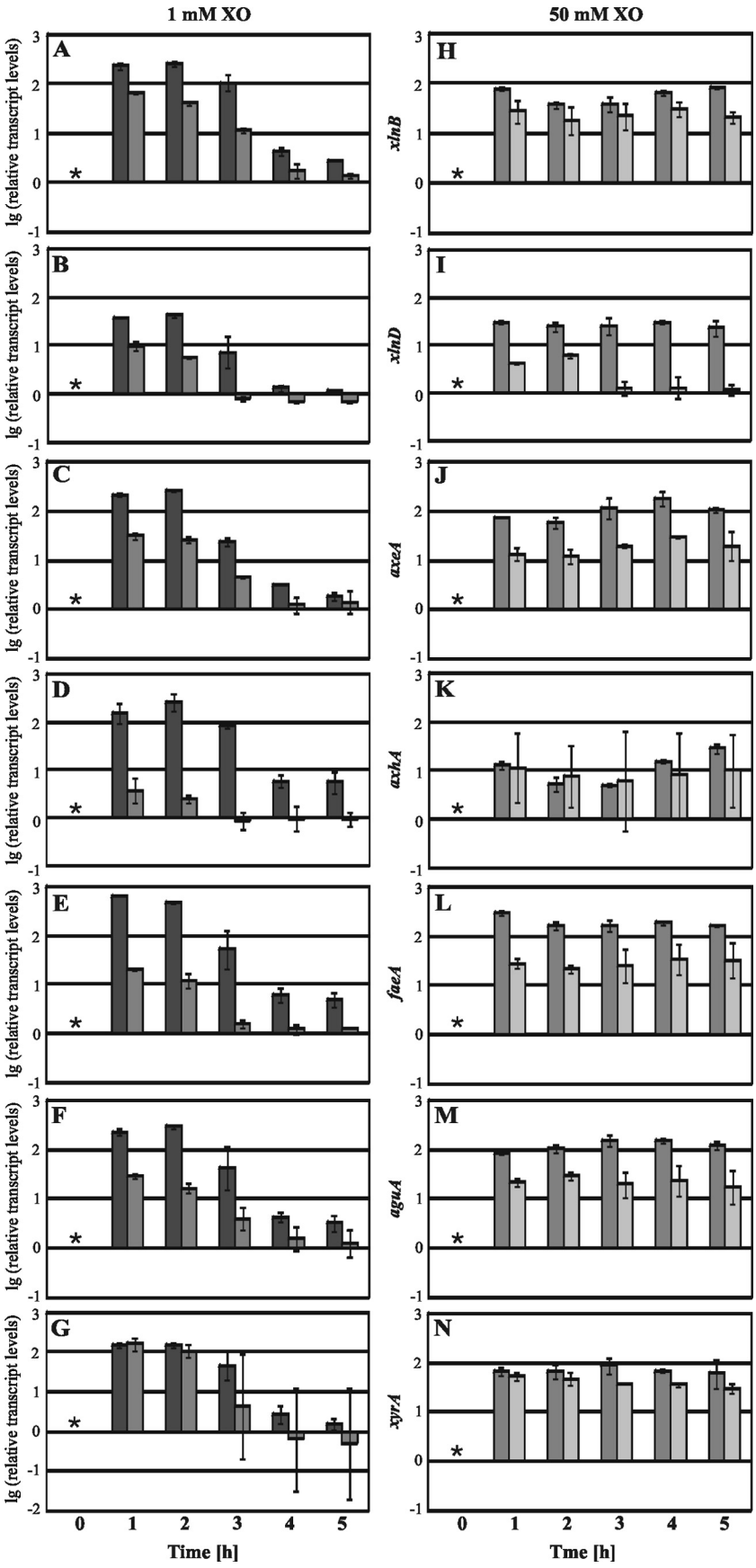


TABLE 4 HPLC analysis of D-xylose and sorbitol concentrations in supernatants of *A. niger* NW283 cultivations in benchtop fermentors

Target D-xylose concn (mM)	Time (h) after induction	Concn (mM)[a]	
		D-Xylose	Sorbitol
1	0	0.9 ± 0.1	90.3 ± 5.8
	1	0.9 ± 0.2	94.3 ± 1.1
	2	0.5 ± 0.1	94.8 ± 2.3
	3	ND[b]	90.8 ± 1.0
	4	ND	91.3 ± 2.7
	5	ND	88.5 ± 3.1
50	0	48.7 ± 4.3	96.1 ± 3.4
	1	47.2 ± 2.8	99.5 ± 5.2
	2	45.6 ± 0.2	96.7 ± 0.6
	3	46.5 ± 1.9	96.0 ± 4.8
	4	45.2 ± 4.0	97.5 ± 6.1
	5	44.2 ± 0.1	95.6 ± 1.0

[a] Values are means and standard deviations from two biological experiments.
[b] ND, not detected; the limit of quantification was <0.03 mM.

whether *xlnR* expression in the two strains was compared (Fig. 6). Briefly, *xlnR* is stable and constitutively expressed, regardless of whether CreA is present and regardless of the amount of D-xylose that is applied.

DISCUSSION

A. niger is an important organism for the production of native enzymes such as hemicellulases. Because D-xylose is often used to induce the expression of hemicellulases, we studied the D-xylose induction kinetics of the system and D-xylose-mediated carbon catabolite repression. In this study, we demonstrated that the use of D-xylose provokes a concentration-dependent response that is mostly specific for genes encoding enzymes with a particular hydrolytic function. Because D-xylose is depleted after 2 h if a concentration of 1 mM is applied for induction, it is most useful to focus on the transcript levels detected in samples taken 1 h after induction for comparison of the inducing potentials of high and low D-xylose concentrations. Nevertheless, we thought that it was also necessary to investigate transcript levels during D-xylose depletion to determine if the constant presence of D-xylose (high concentrations) might be beneficial. At all sampling time points, the cultures were in the log phase with respect to their oxygen consumption.

Although in some cases, the use of 1 mM D-xylose provoked the greatest change in transcript levels, it is important to note that with 50 mM D-xylose, transcript levels that were clearly higher than those from the preinduced samples could be detected. With respect to *xlnB* and *xlnD*, this result is consistent with the study published by de Vries et al. in 1999. In the case of *aguA* and *faeA* with 50 mM D-xylose, hardly any transcript was detected in that study (14), but in the same year, de Vries and Visser observed clear transcript formation of the same genes regardless of whether high (66 mM) or low (2 mM) D-xylose concentrations were applied (13). This discrepancy might be due to the use of Northern blot analysis, which is difficult to standardize. However, the later results are more consistent with the results of this study. Nevertheless, it should also be taken into account that in the present study, all data were obtained similarly during growth, while in the previous studies, growth conditions (high D-xylose concentrations) were compared to cell-resting conditions (low D-xylose concentrations). The experimental design of this study allows a quantitative comparison of 1 and 50 mM D-xylose concentrations, even if rather small differences in transcript levels are observed.

In general, the utilization of a high D-xylose concentration was found to be beneficial, in particular, for the induction of hemicellulase-encoding genes, because their transcript formation is maintained for a longer time. However, *xlnD* clearly poses an exception because its transcript level decreases after 2 h even in the high-D-xylose background. Interestingly, in *A. nidulans*, β-xylosidase activity induced by 66 mM D-xylose was detected after 2 h but decreased afterwards (32). In the same study, a strong *xlnD* transcript was detected after 1 h on D-xylose, which decreased after 2 h (32). However, we observed that the use of a CreA mutant strain completely restored the prolonged transcript formation on 50 mM D-xylose.

The release from CreA influence elevated the induction of the six investigated hemicellulase-encoding genes compared to that in the wild-type strain by 0.5 to 2 orders of magnitude. Notably, this increase was observed independent of whether 1 mM or 50 mM D-xylose was used. Initially, this finding might lead to the conclusion that a relief from carbon catabolite repression provokes a general increase in transcript formation in a sorbitol background. However, a recent study of *A. niger* demonstrated that sorbitol is a noninducing carbon source compared to D-xylose with respect to the expression of hemicellulases and has no inducing effect on the expression of carbohydrate-active enzymes (16). Furthermore, evidence was provided that sorbitol does not repress xylanase expression in *Aspergillus tubingensis* and *A. niger*, while glucose and fructose do repress expression (5). The previously mentioned study by de Vries et al. also reported a D-xylose concentration-independent (ranging from 1 to 100 mM) increase in transcript formation in a CreA mutant, although they transferred the strain to medium containing D-xylose as the only carbon source after pregrowth on fructose (14). Likewise, in *Hypocrea jecorina* (*Trichoderma reesei*), the transfer of a pregrown *cre1* (the *creA* orthologue) deletion strain to D-xylose as the sole carbon source led to increased transcript levels of xylanase-encoding genes compared to those in the parental strain, independent of the D-xylose concentration (26). Taken together, these findings strongly support the assumption that the effects observed in

FIG 5 Influence of CreA on the transcription of genes involved in xylan degradation and D-xylose metabolism in *A. niger*. Strain NW283 (left, dark gray bars) was induced using 1 or 50 mM D-xylose (XO), and samples were taken hourly for 5 h. Transcript level analysis of *xlnB* (A and H), *xlnD* (B and I), *axeA* (C and J), *axhA* (D and K), *faeA* (E and L), *aguA* (F and M), and *xyrA* (G and N) was performed by qPCR using a histone-like gene transcript and a *Schizosaccharomyces pombe dma1*-like gene transcript for normalization. Transcript levels always refer to the reference sample (taken directly after induction) within an experiment, which is indicated by an asterisk. The results are presented as the relative transcript levels in logarithmic scale (lg). The values are means from two independent biological experiments measured in triplicate. Error bars indicate the standard deviations. The transcript levels of the wild-type strain (right, light gray bars) are pictured again for comparison.

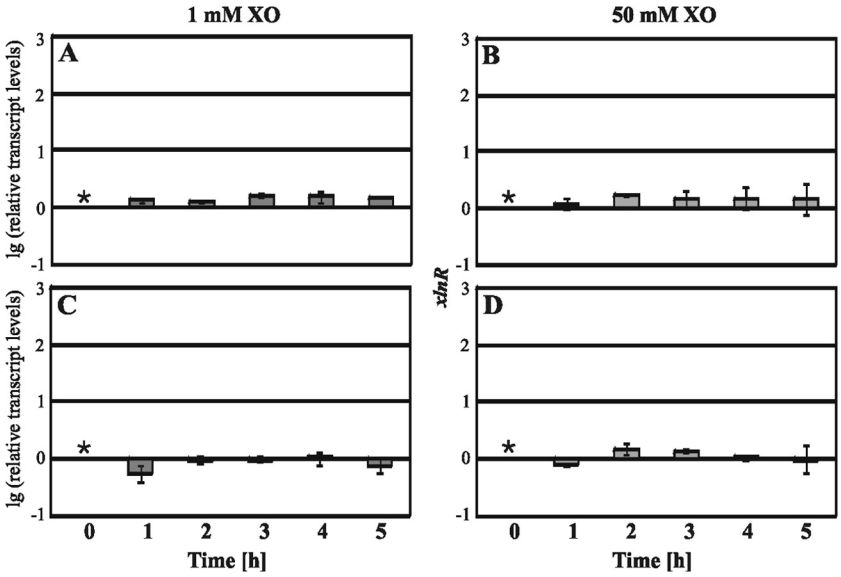


FIG 6 Influence of CreA on the transcription of *xlnR* in *A. niger*. Strains N400 (A and B) and NW283 (C and D) were induced using 1 or 50 mM D-xylose (XO), and samples were taken hourly for 5 h. Transcript level analysis of *xlnR* was performed by qPCR using a histone-like gene transcript and a *Schizosaccharomyces pombe dma1*-like gene transcript for normalization. Transcript levels always refer to the reference sample (taken directly after induction) within an experiment, which is indicated by an asterisk. The results are presented as the relative transcript levels in logarithmic scale (lg). The values are means from two independent biological experiments measured in triplicate. Error bars indicate the standard deviations.

the CreA-deficient strain during this study result from the use of D-xylose as an inducer. Finally, it should be mentioned that *xyrA* was the only gene investigated whose transcript formation did not significantly respond to release from the influence of CreA. Even if there is a single CreA-binding site (CTGGGG) present at a position 757 bp upstream from the *xyrA* structural gene, this does not necessarily mean that it is functional *in vivo* under the conditions used.

Even if the XlnR regulon is inducible by D-xylose, we demonstrated that the transcript levels of *xlnR* do not reflect this induction. This result is consistent with what has been reported for the expression of the XlnR orthologue, Xyr1, from *H. jecorina* (40). In those studies it was found that inducer molecules such as D-xylose, L-arabitol, or xylobiose cannot induce *xyr1* transcription, although they are used to induce xylanase expression (25, 27). Because the induction signal is not mediated/communicated by transcriptional regulation of the *xlnR* gene, XlnR *de novo* synthesis-based regulation or an XlnR-activating posttranslational modification would be a possible alternative regulatory mechanism. Recently, Noguchi et al. provided the first support for such a hypothesis when they reported that D-xylose triggered reversible phosphorylation of XlnR in *Aspergillus oryzae* (28).

Previously, it was reported for *A. nidulans* that CreA represses the expression of *xlnA* and *xlnB* on glucose by an indirect mechanism (29, 30), which was more recently identified as a CreA-mediated repression of the *xlnR* gene (42). Moreover, in *H. jecorina*, repression of *xyr1* transcription by Cre1 on D-glucose as well as on D-xylose was reported (26, 27). However, in this study, we could detect no significant differences in *xlnR* transcript levels in an *A. niger* CreA mutant strain compared to the wild-type strain, regardless of whether low or high D-xylose concentrations were used. This result suggests that other regulatory mechanisms must be acting in the functional regulation of XlnR activity and that they might be present at the posttranscriptional level.

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