

# The Transcriptional Activator XlnR Regulates Both Xylanolytic and Endoglucanase Gene Expression in *Aspergillus niger*

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The expression of genes encoding enzymes involved in xylan degradation and two endoglucanases involved in cellulose degradation was studied at the mRNA level in the filamentous fungus *Aspergillus niger*. A strain with a loss-of-function mutation in the *xlnR* gene encoding the transcriptional activator XlnR and a strain with multiple copies of this gene were investigated in order to define which genes are controlled by XlnR. The data presented in this paper show that the transcriptional activator XlnR regulates the transcription of the *xlnB*, *xlnC*, and *xlnD* genes encoding the main xylanolytic enzymes (endoxylanases B and C and  $\beta$ -xylosidase, respectively). Also, the transcription of the genes encoding the accessory enzymes involved in xylan degradation, including  $\alpha$ -glucuronidase A, acetylxyylan esterase A, arabinoxyylan arabinofuranohydrolase A, and feruloyl esterase A, was found to be controlled by XlnR. In addition, XlnR also activates transcription of two endoglucanase-encoding genes, *eglA* and *eglB*, indicating that transcriptional regulation by XlnR goes beyond the genes encoding xylanolytic enzymes and includes regulation of two endoglucanase-encoding genes.

The two most abundant structural polysaccharides in nature are cellulose and the hemicellulose xylan, which are closely associated in plant cell walls (4). Filamentous fungi, particularly *Aspergillus* and *Trichoderma* species, are well-known and efficient producers of both cellulolytic and hemicellulolytic enzymes. The cellulase degradation system of these organisms consists of three classes of enzymes (2): endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and  $\beta$ -glucosidases (EC 3.2.1.21). Members of all of these classes are necessary to degrade cellulose, a homopolymer of  $\beta$ -1,4-linked D-glucose. Xylan, however, is a heterogeneous polymer with a backbone consisting of  $\beta$ -1,4-linked D-xylose residues, which can be substituted at the C-2 and C-3 positions with various residues, such as acetic acid,  $\alpha$ -L-arabinofuranose, (4-*o*-methyl)glucuronic acid, ferulic acid, and *p*-coumaric acid (5). Due to this heterogeneous composition, a more complex set of enzymes is required for xylan degradation. The following enzymes have been found to be necessary during the cooperative process of xylan breakdown: endoxylanase (EC 3.2.1.8),  $\beta$ -xylosidase (EC 3.2.1.37), acetylxyylan esterase (EC 3.1.1.72),  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55), arabinoxyylan arabinofuranohydrolase,  $\beta$ -glucuronidase (EC 3.2.1.139), feruloyl esterase, and *p*-coumaroyl esterase (3).

The expression of cellulose- and xylan-degrading enzymes by *Aspergillus* and *Trichoderma* species has been studied extensively at the cellular level (1, 20, 21, 25). It has been shown that xylanase- and cellulase-encoding genes are regulated at the transcriptional level (10, 23, 31, 43). In the presence of D-glucose the genes are not expressed, and it has been shown that the carbon catabolite repressor protein CreA is involved in transcriptional repression of xylanase-encoding (10) and arabinase-encoding (38) genes in *Aspergillus* species. It has been demonstrated that in *Trichoderma reesei* the CreA counterpart

Cre1 causes repression of transcription of cellulase-encoding (22, 23) and xylanase-encoding (30, 31) genes. However, far less is known about the mechanism by which cellulase- and xylanase-encoding genes are induced. The inducing abilities of various saccharides have been tested, and some saccharides induce the synthesis of both xylanases and cellulases (10, 21, 31, 37, 48). Nevertheless, on the basis of biochemical data (1, 20, 21) and mRNA expression analysis data (23, 31), a separate induction mechanism has been proposed for these systems in both *Aspergillus* and *Trichoderma*.

Recently, a selection system was developed to isolate *Aspergillus niger* strains having mutations in a transcription factor involved in induction of expression of xylanolytic genes. Complementation of such a mutation by transformation with a plasmid library led to the isolation of the *A. niger xlnR* gene, which encodes a transcriptional activator of the *A. niger* xylanolytic system (44). This *xlnR* gene encodes a zinc binuclear cluster protein, which is a member of the GAL4 family of transcription factors. Isolation of both the *xlnR* gene and *A. niger xlnR* loss-of-function mutants provided an opportunity to study the spectrum of genes that are controlled by XlnR at the transcriptional level.

## MATERIALS AND METHODS

***Aspergillus* strains, transformation, and culture conditions.** All of the *A. niger* strains used were derived from wild-type strain N400 (= CBS 120.49). The strains used were *A. niger* N402 (*cspA1*), a short-conidiophore derivative, NW205::130 [*argB13 cspA1 nicA1 pyrA6 UAS(xlnA)-pyrA*], NXA1-4 [*argB13 cspA1 nicA1 pyrA6 UAS(xlnA)-pyrA xlnR1*] (strains NW205::130 and NXA1-4 are described more extensively in reference 44), and N902 (*argB15 cspA1 fwnA1 metB10 pyrA5*).

Strain N902::230-25.12 (*argB15 cspA1 fwnA1 metB10*), which contains approximately 20 additional copies of *xlnR*, as determined by a phosphorimager analysis of Southern blots, was obtained by cotransformation of *A. niger* N902. The cotransforming plasmids were pIM230 (44) and pGW635 (19), which contain the functional *xlnR* gene (EMBL accession no. AJ001909) and the *pyrA* gene (EMBL accession no. X96734), respectively. Transformation was carried out as described previously (27).

All media were based on *Aspergillus* minimal medium (36). The media contained the carbon sources indicated below, and the starting pH of each medium was 6. Spores were inoculated at a concentration of  $10^6$  spores ml<sup>-1</sup>. In transfer experiments the first culture containing D-fructose was supplemented with 0.2% (wt/vol) Casamino Acids and 0.1% (wt/vol) yeast extract. After overnight growth,

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TABLE 1. Probes used in Northern blot analysis

Gene	EMBL accession no.	Enzyme encoded	Fragment used	Reference
<i>abfB</i>	X74777	$\alpha$ -L-Arabinofuranosidase B	1.7-kb <i>EcoRI-XhoI</i>	15
<i>aguA</i>	Y15405	$\alpha$ -Glucuronidase A	0.8-kb <i>EcoRV-KpnI</i> <sup>a</sup>	12
<i>axeA</i>	A22880	Acetyl xylan esterase A	1.5-kb <i>HindIII</i> <sup>a,b</sup>	9
<i>axhA</i>	Z78011	Arabinoxylan hydrolase A	1.2-kb <i>EcoRI-XhoI</i>	17
<i>bglA</i>		$\beta$ -Glucosidase A	1.0-kb <i>NcoI-SstI</i>	This study
<i>eglA</i>	AJ224451	Endoglucanase A	0.9-kb <i>XhoI</i>	This study
<i>eglB</i>	AJ224452	Endoglucanase B	1.1-kb <i>EcoRI-XhoI</i>	This study
<i>faeA</i>	Y09330	Feruloyl esterase A	0.5-kb <i>EcoRV-XhoI</i> <sup>a</sup>	11
<i>xlnB</i>	D38071	Endoxylanase B	0.9-kb <i>EcoRI-XhoI</i>	24
<i>xlnC</i>		Endoxylanase C	1.2-kb <i>EcoRI-XhoI</i>	18
<i>xlnD</i>	Z84377	$\beta$ -Xylosidase D	2.8-kb <i>PstI-NsiI</i> <sup>a</sup>	43
18S rRNA	X78538	18S rRNA subunit	0.7-kb <i>EcoRI</i>	33

<sup>a</sup> Genomic fragment instead of cDNA.

<sup>b</sup> Fragment from the *Aspergillus tubingensis axeA* gene.

mycelia were recovered by filtration and washed with saline. These mycelia were transferred to media containing D-xylose or xylan as a carbon source and 0.05% (wt/vol) Casamino Acids. The xylan used was birchwood xylan (Roth-7500).

**Expression cloning of *A. niger* glucanases in *Escherichia coli*.** A xylan-induced cDNA library of *A. niger* (43) was screened for expression of endoglucanases by using a modified procedure (6, 46, 47). The plates contained 20 ml of 2 $\times$  TY, 0.2% carboxymethyl cellulose (CMC) (Sigma), 1.5% agar, and 100  $\mu$ g of ampicillin per ml. *E. coli* cells were plated in an overlay consisting of 5 ml of the same medium containing about 300 colonies per plate, and the plates were incubated for 48 h at 37°C. Next, 5 ml of 0.1% Congo red (Aldrich) was poured onto each plate. After it was stained for 1 to 2 h, each plate was destained with 5 ml of 5 M NaCl for 0.5 to 1 h. About 12,000 colonies from the *A. niger* cDNA library were plated. Screening on CMC resulted in 89 colonies that had halos after staining with Congo red. None of these colonies produced a halo when it was screened with Remazol brilliant blue-modified xylan. All colonies contained a full-length cDNA copy, which appeared to originate from two different genes. Both of the enzymes encoded were active on CMC and on  $\beta$ -glucan (unpublished data). The corresponding genes, *eglA* and *eglB*, were cloned by using these cDNA fragments.

**Northern blot analysis.** Total RNA was isolated from powdered mycelia by using TRIzol reagent (Life Technologies) according to the supplier's instructions. For Northern blot analysis 10  $\mu$ g of total RNA was glyoxylated and separated on a 1.6% (wt/vol) agarose gel (39). After capillary blotting onto Hybond-N filters (Amersham), the amounts of RNA were checked by staining the rRNA on the Hybond filters with a 0.2% (wt/vol) methylene blue solution. The filters were hybridized at 42°C in a solution containing 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, 0.9 M NaCl, 90 mM trisodium citrate, 0.2% (wt/vol) Ficoll, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) bovine serum albumin, 0.1% (wt/vol) sodium dodecyl sulfate, and 100  $\mu$ g of single-stranded herring sperm DNA per ml. Washing was done under homologous hybridization conditions with a solution containing 30 mM NaCl, 3 mM trisodium citrate, and 0.1% (wt/vol) sodium dodecyl sulfate at 68°C. The <sup>32</sup>P-labelled DNA probes used were either cDNA or genomic fragments, as shown in Table 1.

A 1-kb  $\beta$ -glucosidase cDNA fragment of *A. niger*, as determined by sequence analysis, was isolated from a xylan-induced cDNA library (43) by using PCR with degenerate oligonucleotides based on the *Aspergillus kawachii* (EMBL accession no. AB003470) and *Aspergillus aculeatus* (EMBL accession no. P48825) sequences for  $\beta$ -glucosidase and cloned into pGEM-T (Promega).

**Nucleotide sequence accession numbers.** The *eglA* and *eglB* sequences have been deposited in the GenBank-EMBL sequence database under accession no. AJ224451 and AJ224452, respectively.

## RESULTS AND DISCUSSION

**Induction of the xylanolytic system.** An *A. niger* mutant having a loss-of-function mutation in the xylanolytic transcriptional activator gene *xlnR* lacks transcription of the endoxylanase B- and  $\beta$ -xylosidase-encoding genes *xlnB* and *xlnD* (44). To investigate the spectrum of genes which are under control of the transcriptional activator *xlnR*, expression in an *A. niger* wild-type strain and expression in the strain with the *xlnR* loss-of-function mutation were analyzed by Northern blot analysis. To do this, we used fragments of genes cloned from *A. niger* encoding enzymes which are potentially involved in the breakdown of xylan (Table 1). *A. niger* NW205::130 (wild type) and NXA1-4 (a *xlnR* mutant) were precultured and subsequently

transferred to media containing 1% birchwood xylan and to media containing 1% D-xylose (both birchwood xylan and D-xylose are known to be carbon sources that induce the xylanolytic system in *A. niger*) (10). Northern blot analysis of RNA obtained from the wild-type strain showed that xylanolytic, arabinolytic, and cellulolytic genes were induced when the organism was grown on xylan, which is the natural substrate, and on D-xylose (Fig. 1A). High levels of expression were obtained in most cases after 6 h of growth on the polymeric carbon source xylan, although the patterns of expression for individual genes differed. Whereas some genes, including *xlnD* and *aguA*, had a high transcription level during the early phase of induction, other genes, including *axeA* and *eglB*, were highly transcribed at a later stage. The level of induction on D-xylose was usually lower than the level of induction on xylan, but some genes, including *xlnD* and *xlnB*, had a relatively high level of expression on D-xylose. As expected for extracellular enzyme systems under the control of carbon catabolite repression (10, 38), none of the genes was expressed on D-fructose.

**Effect of the *xlnR* loss-of-function mutation on expression.** An analysis of transcription in the *xlnR* loss-of-function mutant NXA1-4 revealed expression of only *abfB* and *bglA* upon growth on D-xylose and xylan (Fig. 1A). Mutant NXA1-4 lacks the ability to induce transcription of genes encoding xylanolytic enzymes which are involved in the degradation of the polyxylose backbone of xylan. Also, transcription of genes encoding accessory enzymes is absent in this mutant. These findings explain the previously described impaired growth of NXA mutants on xylan (44). Strains with an *xlnR* loss-of-function mutation are not able to express the xylanolytic enzymes, which results in impaired release of saccharides (and therefore carbon source) from the polymeric xylan. Although arabinofuranosidase B is expressed in these mutants, apparently the L-arabinose released from arabinoxylan by this enzyme is not sufficient to allow normal growth of the fungus. The inability of the *xlnR* loss-of-function mutants to degrade xylan also affects the release of inducer from the polymeric substrate. Induction by D-xylose, however, is independent of the presence of the xylanolytic enzyme system. Therefore, gene expression was reexamined in a second experiment by using mutant NXA1-4, wild-type strain N902, and *xlnR* multicopy strain N902::230-25.12; D-xylose was used as the inducing carbon source in this experiment.

**Effect of multiple copies of *xlnR*.** In the wild-type strain all of the genes tested were induced on D-xylose (Fig. 1B), whereas in strain NXA1-4 only *abfB* and *bglA* transcription was observed. In *xlnR* multicopy strain N902::230-25.12 all of the

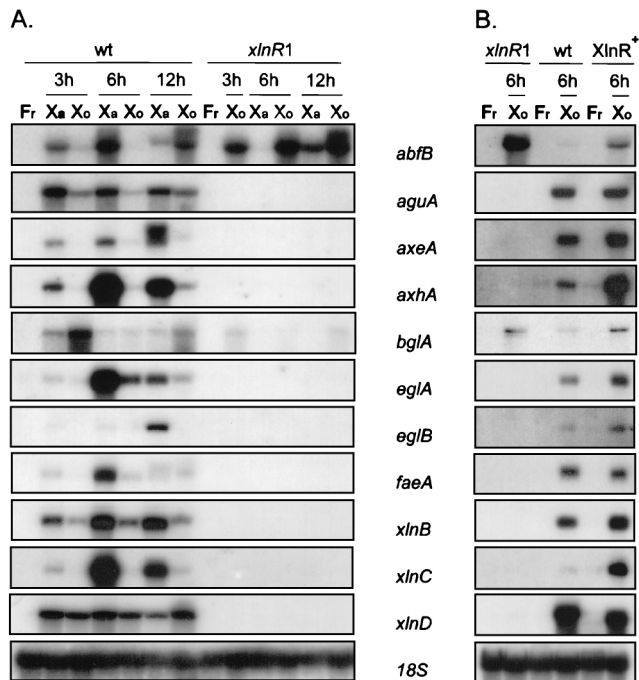


FIG. 1. Northern blot analysis of expression of *A. niger* genes encoding cellulose- and xylan-degrading enzymes. (A) Time course of induction in *A. niger* NW205::130 (wt) and NXA1-4 (*xlnR1*) (a loss-of-function mutant). Both strains were cultured for 18 h in medium containing 3% D-fructose (Fr), and mycelia were subsequently transferred to medium containing 1% xylan (Xa) or 1% D-xylose (Xo) and incubated for the times indicated. Each lane contained 10  $\mu$ g of total RNA, which was checked by hybridization with the 18S rRNA probe. Blots were hybridized with gene-specific probes as indicated. (B) Comparison of expression of genes encoding cellulose- and xylan-degrading enzymes in *A. niger* N902 (wt), NXA1-4 (*xlnR1*), and N902-pIM230-25.12 (XlnR<sup>+</sup>) (N902 with multiple copies of *xlnR*) upon transfer to medium containing 1% D-xylose for 6 h after growth for 18 h in medium containing 1% D-fructose. A Northern blot analysis was performed exactly as described above. The signal intensities of the different blots cannot be compared to each other due to the unknown specific activities of the probes used and the different exposure times used for the various blots.

genes were also expressed. Some genes (for example, *aguA* and *faeA*) had equal transcript levels in both the wild-type and the *xlnR* multicopy strain, whereas other genes (for example, *abfB*, *axhA*, *bglA*, *xlnB*, and *xlnC*) had increased transcription levels in the *xlnR* multicopy strain compared to that in the wild-type strain. From this finding we concluded that the transcriptional activator XlnR regulates the transcription of the *xlnB*, *xlnC*, and *xlnD* genes encoding the main xylanolytic enzymes (endoxyylanases B and C and  $\beta$ -xylosidase, respectively). In addition, the *aguA*, *axeA*, *axhA*, and *faeA* genes encoding accessory enzymes ( $\alpha$ -glucuronidase A, acetylxyylan esterase A, arabinoxyylan arabinofuranohydrolase A, and feruloyl esterase A) are controlled by the transcriptional regulator XlnR. The transcriptional activator XlnR also activates transcription of the *eglA* and *eglB* genes, which encode endoglucanases A and B. This indicates that regulation by the transcriptional activator XlnR goes beyond regulation of the genes encoding xylanolytic enzymes and also includes regulation of at least two endoglucanase-encoding genes.

All of the genes that were found to be controlled by the transcriptional activator XlnR exhibited differences in their levels of expression in response to increased *xlnR* gene copies. The differences in the responses to the *xlnR* gene copy number might originate from differences in the XlnR binding sites in the various xylanolytic promoters. The sequence 5'-GGCTAA

A-3' has been suggested previously to be a consensus binding site for the XlnR protein; this suggestion was based on the results of a comparison of a limited number of mainly endoxyylanase promoters of different *Aspergillus* species (44). The results presented here show that expression of at least nine genes in *A. niger* is controlled by XlnR. The *axeA*, *axhA*, *eglA*, *eglB*, and *xlnC* genes, for which an increased *xlnR* copy number has a positive effect on the level of transcription, all have a nucleotide other than adenine at the last position. Transcription of *xlnB*, which has an adenine at the last position, however, is also positively influenced. A comparison of the sequences of these nine promoters (Table 2) suggests, therefore, that the last nucleotide in the proposed consensus sequence is less important in the binding site and that 5'-GGCTAA-3' is a more appropriate consensus sequence, but the seventh nucleotide could play a role in XlnR binding.

All of the *A. niger* genes for which XlnR transcriptional control has been demonstrated contain one or more copies of this consensus sequence in the promoter region. The different genes vary in the number of putative XlnR binding sites present, as four genes have two putative sites. Also, the orientation varies, as some genes have the opposite orientation or both orientations are present. The differences found in the effect of the *xlnR* copy number and the level of transcription of the individual genes cannot be explained by the differences in the presumed XlnR binding sites, since the mode of binding of XlnR is not known (44).

The context in which the sites are located in the promoter region may also play an important role. The putative XlnR binding site is not a direct or inverted repeat, while most zinc binuclear cluster proteins have a dimeric nature and bind to symmetric sites. However, some proteins (for example, the AlcR protein of *Aspergillus nidulans*) are thought to act as monomers. Two molecules of AlcR can simultaneously bind to symmetric sites, whereas only one molecule occupies a direct repeat (28).

It has been proposed that repression by CreA of the xylanolytic genes (10, 44) is analogous to the double lock mechanism described for the ethanol regulon in *A. nidulans* (13, 26). In this model CreA represses both the positive and autoregulated *trans*-acting gene *alcR* and structural genes, such as *alcA* and *aldA* (14, 26, 28, 32). Some CreA binding sites in the *alcA* and *alcR* upstream region are close to or overlap the AlcR targets (13, 26). Therefore, it has been suggested that competition between the AlcR and CreA proteins for the same region is a mechanism in the regulation of the ethanol regulon genes. This is also the case in the regulation of expression of *amdS* by the

TABLE 2. Putative XlnR binding sites in the upstream region of XlnR-controlled genes

Gene	XlnR binding site	Position(s) <sup>a</sup>
<i>aguA</i>	GGCTAAa	-276
<i>axeA</i>	GGCTAAat	-261 (R)
<i>axhA</i>	GGCTAAat	-340
	GGCTAAg	-850 (R)
<i>eglA</i>	GGCTAAg	-710
<i>eglB</i>	GGCTAAg	-128
<i>faeA</i>	GGCTAAa	-265, -225
<i>xlnB</i>	GGCTAAa	-124, -216
<i>xlnC</i>	GGCTAAat	-290
	GGCTAAg	-500
<i>xlnD</i>	GGCTAAa	-133, -147

<sup>a</sup> Position relative to the ATG translation start codon. (R) indicates the opposite orientation of the putative XlnR binding site.

*trans*-acting factors AmdR, FacB, AmdA, AmdX, AreA, and CreA (8, 29, 34, 35). The overlap of AmdX binding sites with CreA and AmdA binding sites suggests that there is competition for binding sites by multiple factors (34). The repressor protein CreA has been shown to also have a function in xylanolytic gene expression in *A. niger* (10, 17). However, putative CreA sites in the XlnR-controlled genes in *A. niger* are generally at distances of more than 40 bp from the putative XlnR binding sites; an exception is the 1-bp distance in the *xlnD* upstream region (43). Thus, XlnR-CreA competition for all xylanolytic promoters is unlikely. Besides the *trans*-acting factors XlnR and CreA, other *trans*-acting factors may have a function in modulating the transcription of the various XlnR-controlled genes.

Transcription of the  $\beta$ -glucosidase-encoding gene *bglA* is under separate control, and therefore not all genes encoding cellulolytic enzymes are controlled by XlnR. Of the genes involved in xylan degradation, the *abfB* gene is the only gene whose transcription is not controlled by XlnR. The encoded enzyme, however, is involved in hydrolysis of L-arabinofuranosyl residues not only from arabinoxylan but also from arabinan (41) and pectin (40). *abfB* gene expression is under coordinate control with expression of the arabinofuranosidase A-encoding *abfA* gene and the endoarabinase-encoding *abnA* gene (16). Although it is clear from the results presented here that the *abfB* and *bglA* genes are not controlled by XlnR, the level of transcription is increased in both the NXA1-4 mutant and the *xlnR* multicopy transformant. The promoter sequence of the *A. niger bglA* gene is not available, and the *abfB* gene does not contain the XlnR binding site. The increase in the levels of expression of *abfB* and *bglA* on D-xylose may be an indirect effect of the *xlnR* loss-of-function mutation and gene dosage. For example, there could be an effect on pentose catabolism (45), thereby influencing the L-arabitol concentration, which is the inducer of the *abfB* gene (42).

The use of a loss-of-function mutation in the transcriptional activator XlnR is a powerful tool for understanding the fungal strategy for degrading the variety of xylan structures which occur in nature. The fact that expression of the xylanolytic enzymes and expression of some cellulolytic enzymes are coordinately regulated at the molecular level provides new insight into the regulation of expression of both enzyme systems. The findings presented here strengthen the hypothesis that there is an evolutionary relationship between some of the xylanolytic and cellulolytic enzyme systems. Xylanases and cellulases have been shown to be related at various levels. The three-dimensional structures of, for example, family 11 endoxylanases and family 12 endoglucanases are similar (7). Also, there are similarities in the primary structures of, for example,  $\beta$ -xylosidase XlnD and  $\beta$ -glucosidase BglA, both of which are members of the family 3 glycosyl hydrolases (43). Here we provide evidence that there is coordination in the regulation of xylanases and some cellulases.

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