Transcriptional activator, AoXlnR, mediates cellulose-inductive expression of the xylanolytic and cellulolytic genes in *Aspergillus oryzae*

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Abstract AoXlnR was isolated as a transcriptional activator of the major xylanase gene, *xynF1*, in *Aspergillus oryzae*. To investigate the spectrum of genes under the control of AoXlnR, expression of the xylanolytic and cellulolytic genes in an *A. oryzae* wild type strain, an *AoxlnR* disruptant and an *AoXlnR* overexpressed strain was analyzed by Northern blotting. AoXlnR mediated expression of at least four xylanolytic genes and four cellulolytic genes when induced by xylan and D-xylose. Moreover, AoXlnR was newly found to mediate the cellulose-inductive expression of the xylanolytic genes as well as the cellulolytic genes.

Key words: Xylanolytic genes; Cellulolytic genes; *Aspergillus oryzae* XlnR; *Aspergillus oryzae*

1. Introduction

Cellulose and xylan, which are closely linked together in plant cell walls [1], are abundant and renewable polysaccharides. Enzymatic degradation of these polysaccharides attracts attention with respect to biotechnological applications. Filamentous fungi produce a complex spectrum of enzymes to degrade cellulose and xylan. A number of genes encoding the cellulolytic and xylanolytic enzymes have been isolated from various filamentous fungi, and the regulatory machinery of these genes have been investigated extensively in *Aspergillus* and *Trichoderma* species. Recently, a transcriptional activator, XlnR, was cloned from *Aspergillus niger* [2]. XlnR mediates the xylan-dependent expression of the xylanolytic genes as well as the cellulolytic genes [2–5]. In addition, transcription of the α-galactosidase B-, β-galactosidase A- and D-xylose reductase-encoding genes is controlled by XlnR [6,7].

The *Aspergillus oryzae xlnR* (*AoxlnR*) gene, a homolog of the *A. niger xlnR* gene, has also been shown to mediate the xylan-dependent expression of the xylanolytic genes such as the *xynF1* and *xylA* genes in *A. oryzae* [8]. To further examine the spectrum of the xylanolytic and cellulolytic genes under the control of AoXlnR, their expression in an *A. oryzae* wild type (WT) strain, an *AoxlnR* disruptant and an *AoxlnR* overexpressed strain was analyzed by Northern blotting. Interestingly, it is the first to show that AoXlnR mediated the cellulose-dependent expression of the xylanolytic genes as well as the cellulolytic genes in *A. oryzae*. This was further confirmed by monitoring β-galactosidase (β-Gal) activity in transformants carrying the authentic or mutated *xynF1* promoter controlled *E. coil lacZ* gene grown on cellulose.

2. Materials and methods

2.1. Strains, plasmids, media and transformation

A. oryzae KBN616, an industrial shoyu koji mold obtained from bio'c (Toyohashi, Japan), was used to isolate DNA. *A. oryzae* P3, a uridine-requiring host derived from *A. oryzae* KBN616, was used for transformation. *A. oryzae* WTZ, Mu1Z, Mu3Z and Mu5Z strains carrying a single copy of the authentic or mutated *xynF1* promoter-controlled *lacZ* gene were previously constructed [8]. *Escherichia coli* XL1-Blue (Stratagene) was used for DNA manipulation. Plasmids, pUC and pBluescript II KS (+), were used to subclone various DNA fragments. Czapek-Dox medium consisting of 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$ (pH 6.5) was used as the minimal medium for fungal growth and supplemented with various carbon sources in each experiment. Strains of plasmid-carrying *Escherichia coli* were grown at 37°C in LB medium containing 50 μg/ml of ampicillin. Transformation of *E. coli* was performed by the method of Hanahan. Transformation of *A. oryzae* P3 was performed as described by Ballance and Turner [9]. Protoplast was prepared from *A. oryzae* P3 using Novozyme234 (Calbiochem). A transformant obtained by introducing plasmid pYRG100 carrying the *A. oryzae pyrG* gene [8] was used as a WT strain for Northern analysis.

2.2. Construction of the AoxlnR disrupted strain

An *AoxlnR* disruption plasmid pDisXR100 was constructed as follows. A *Nru*I fragment of the *AoxlnR* gene from +151 to +1779 (referring to the translation start site as +1) was isolated by digestion of pAX2 carrying a 2.6 kb *Pst*I–*Hin*dIII fragment of the *AoxlnR* gene [8], followed by insertion to the *Sma*I site of plasmid pYRG100 carrying the *A. oryzae pyrG* gene (plasmid pDisXR100). Protoplasts were mixed with pDisXR100 and *Sph*I to carry out the restriction enzyme-mediated integration. One of the transformants, *A. oryzae* SK253, which grew very poorly on xylan while it grew as well as the WT on D-glucose [8] was used for further analysis. Disruption of the endogenous *AoxlnR* gene was confirmed by Southern blot analysis.

2.3. Construction of the AoxlnR overexpressed strain

The overexpression plasmid pTFXR100 was constructed as follows. The *AoxlnR* gene was amplified by PCR with Ex-Taq (Takara) using a pair of primers, TXR-S (5′-GGATGCATATGTCGACGACCTCGATTCAG-3′) corresponding to +1 to +21 and TXR-AS (5′-GGTCTAGATGCGCATATATCATGTC-3′) corresponding to +3652 to +3674 referring the translation start site as +1. A genomic DNA of *A. oryzae* KBN616 was used as template. An *Eco*T22I site was introduced just before the ATG codon of the *AoxlnR* gene in order to

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ligate the *TEF1* promoter fragment precisely next to the *AoxlnR* coding region. Then, a 3.6-kb *Eco*T22I–*Xba*I *AoxlnR* DNA fragment was inserted to the *Eco*T22I–*Xba* I site on pYRM200 carrying *TEF1* promoter derived from pTF100 [10] and the *pyrG* gene to create pTFXR100, followed by introduction into *A. oryzae* P3 as described above. One of the transformants, *A. oryzae* TFX2, with the highest xylanase activity on xylan was used for further analysis. All the amplified DNA fragments by PCR were sequenced to confirm their identity. Construction of pYRM200 will be published elsewhere.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs isolated from *A. oryzae* WT strain (WT) and *AoxlnR* overexpressed strain (HxlnR) cultured for 24 h at 30°C in minimal medium supplemented with 1% polypeptone were treated with DNase, and then 5 μg of RNAs was used for cDNA synthesis by Super script first-strand synthesis system for RT-PCR (Invitrogen) with oligo-dT primers. cDNA fragments of the *AoxlnR* gene and the *gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase (accession number: AB032274) were amplified by limiting the cycle numbers (10, 20 and 30) of the PCR using Ex-Taq (Takara) and oligonucleotide primers (*AoxlnR*, sense primer 5′-AGTGTACAGGACACAGCATGTTCG-3′ and antisense primer 5′-TAGTGCAAGACCACTGCCATC-3′; *gpdA*, sense primer 5′-TACATCGTCGAGTCCACTGGT-3′ and antisense primer 5′-GTAGGCAATGAGGTCAACAACAC-3′).

2.5. Northern blot analysis

20 μg of total RNAs prepared from various *A. oryzae* strains using a Trizol reagent (Invitrogen) was separated on a formaldehyde–agarose gel [11], and then transferred to a Hybond-N$^+$ membrane (Amersham Pharmacia). ^{32}P-labeled DNA probes were prepared as described previously [8]. Hybridization was carried out at 42°C in the ULTRAhyb (Ambion) hybridization buffer. The membranes were washed following manufacturer's recommendation and autoradiographed. The DNA fragments used as probe were described in Table 1.

2.6. Other methods

Genomic DNAs were extracted from various *A. oryzae* strains using an ISOPLANT (Nippon gene). β-Gal activity in mycelial extracts was measured as described previously [8]. 1 U of β-Gal was defined as the amount of the enzyme, which hydrolyzes 1 nmol of *o*-nitrophenyl β-D-galactopyranoside to *o*-nitrophenol and D-galactose per minute. Recombinant DNA techniques were carried out by standard procedures [11]. Nucleotide sequences were determined as described previously [8].

3. Results and discussion

3.1. Xylan-dependent induction of the xylanolytic and cellulolytic genes

The XlnR consensus binding sequence (5′-GGCTA/GA-3′) [3,8] and its analogous sequence (5′-GGCTAT-3′) were widely found in the promoter regions of the xylanolytic and cellulolytic genes in *A. oryzae* (Fig. 1). AoXlnR has been shown to mediate expression of the *xynF1* and *xylA* genes in *A. oryzae* [8]. To further demonstrate that AoXlnR could regulate many other xylanolytic and cellulolytic genes, their expression in an *A. oryzae* WT strain, an *AoxlnR* disruptant and an *AoxlnR* overexpressed strain was analyzed by Northern blotting.

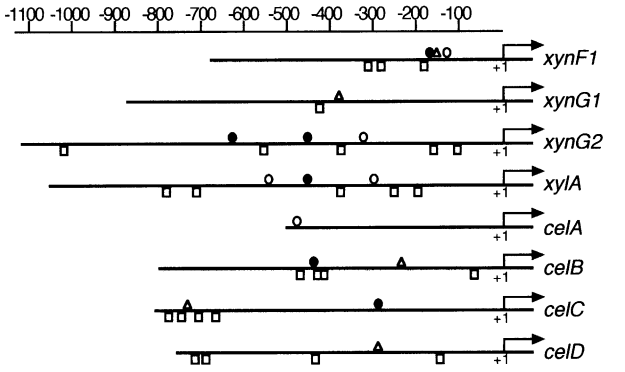

Fig. 1. Localization of putative XlnR and CreA binding sequences in the promoter regions of the xylanolytic and cellulolytic genes in *A. oryzae*. The high-affinity XlnR binding sequence (5′-GGCTAA-3′), low-affinity binding sequence (5′-GGCTGA-3′) [3,8] and their analogous sequences (5′-GGCTAT-3′) are indicated by filled circles, open circles and open triangles, respectively. Putative CreA binding sequences (5′-SYGGRG-3′) [19,20] are indicated by open squares. The deduced translation start site in each gene is indicated as +1.

The *AoxlnR* gene was shown to be expressed constitutively at a low level in the *A. oryzae* WT strain by RT-PCR (Fig. 2). This low endogenous expression necessitated use of a stronger constitutive promoter for AoXlnR. The *AoxlnR* gene was constitutively overexpressed under the control of the *A. oryzae* TEF1-α gene (*TEF1*) promoter [10] (Fig. 2).

As shown in Fig. 3A, all the xylanolytic genes and the *celB* and *celC* genes were induced by xylan and D-xylose in the WT strain. By contrast, no transcripts of these genes were detected in the *AoxlnR* disrupted strain under the same growth conditions. Expression of all the xylanolytic and cellulolytic genes including the *celA* and *celD* genes of which transcripts were not detected even in the WT strain was induced markedly in the *AoxlnR* overexpressed strain. Enhancement of the xylan-inductive expression of the xylanolytic and cellulolytic genes bearing XlnR consensus binding sequence was correspondingly observed in *A. niger* multicopy strains [3–5,7]. These results clearly indicate that AoXlnR mediates the xylan-dependent induction of the xylanolytic and cellulolytic genes in *A. oryzae* in the same manner as described for *A. niger*.

3.2. Involvement of AoXlnR in cellulose-dependent induction of the xylanolytic and cellulolytic genes

Cellulose and its derivatives have been shown to induce expression of the xylanolytic genes as well as the cellulolytic

Table 1
Probes used in Northern blot analysis

Gene	Enzyme encoded	Fragment used	Accession number
xynF1	endoxylanase F1	1.4 kb *Sal*I–*Cla*I	AB011212
xynG1	endoxylanase G1	0.6 kb *Sac*I–*Kpn*I	AB003085
xynG2	endoxylanase G2	0.7 kb *Bst*XI–*Hin*cII	AB044941
xylA	β-xylosidase A	1.6 kb *Kpn*I–*Sma*I	AB013851
celA	endoglucanase A	1.2 kb *Xho*I–*Eco*RI	D83731
celB	endoglucanase B	1.6 kb *Eco*T22I–*Sma*I	D83732
celC	cellobiohydrolase C	1.6 kb *Bgl*II	AB089436
celD	cellobiohydrolase D	1.8 kb *Pst*I–*Pvu*I	AB089437
16S rRNA	mitochondrial 16S rRNA	0.3 kb *Kpn*I–*Sac*I[a]	J01390

[a]DNA fragment from the *Aspergillus nidulans* rRNA gene.

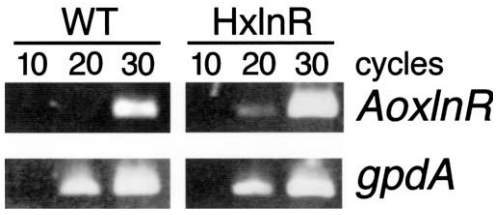

Fig. 2. The expression of the *AoxlnR* gene in *A. oryzae* WT and *AoxlnR* overexpressed strains. The expression of the *AoxlnR* gene in *A. oryzae* WT strain and *AoxlnR* overexpressed strain (HxlnR) cultured with 1% polypeptone was analyzed by limiting the cycle numbers (10, 20 and 30) of the RT-PCR as described in Section 2. The mRNA of the *gpdA* gene was used as an internal control.

genes in both *Trichoderma reesei* [12–17] and *Aspergillus terreus* [18]. However, it has not yet been clarified that XlnR or its homologs mediate their expression. We postulated that AoXlnR might also mediate the cellulose-dependent induction of the xylanolytic and cellulolytic genes in *A. oryzae*, since AoXlnR was absolutely required for their expression on xylan. RNAs were prepared from the *A. oryzae* WT strain, the *AoxlnR* disrupted strain and the *AoxlnR* overexpressed strain grown on Avicel (microcrystalline cellulose) or cellobiose, and subjected to Northern blot analysis (Fig. 3B). Surprisingly, the xylanolytic and cellulolytic genes examined here were induced

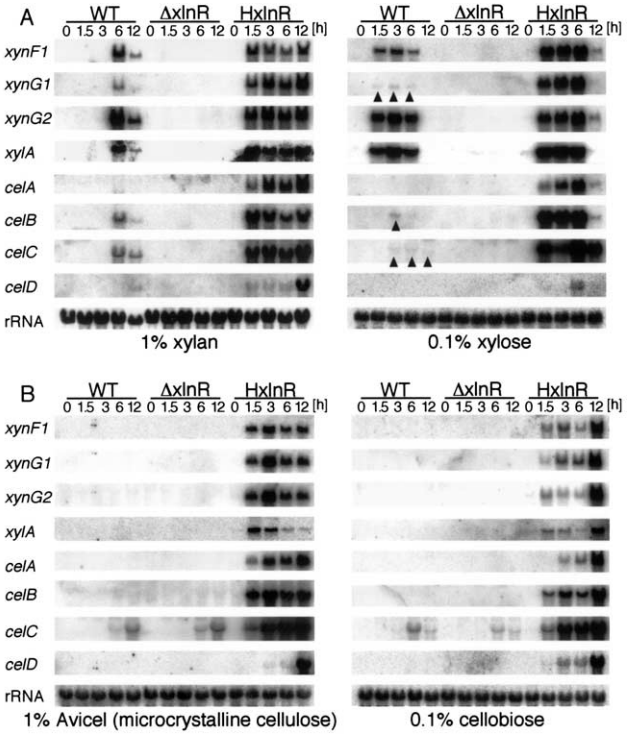

Fig. 3. Northern blot analysis of expression of the xylanolytic and cellulolytic genes in *A. oryzae* grown on xylan or D-xylose (A), and on Avicel or cellobiose (B). *A. oryzae* WT strain, *AoxlnR* disrupted strain (ΔxlnR) and overexpressed strain (HxlnR) were grown for 24 h at 30°C in minimal medium supplemented with 1% polypeptone. Mycelia recovered by filtration were washed with minimum medium and transferred to minimal medium containing 1% xylan or 0.1% D-xylose (A), and 1% Avicel or 0.1% cellobiose (B) as an inducing carbon source followed by incubation for the time indicated. Blots were hybridized with gene-specific probes as indicated. A 16S mitochondrial rRNA was used as an internal marker. The arrowheads indicate weak hybridization signals.

efficiently in the *AoxlnR* overexpressed strain on Avicel. All the genes except for the *celD* gene were expressed within 1.5 h of induction. This was also the case when cellobiose was used as an inducer. However, the expression levels of these genes except for the *celC* gene remained low within 1.5–6 h of induction. Cellobiose might be rapidly degraded to glucose, which could in turn excert the carbon catabolite repression on them, since putative CreA binding sites (5′-SYGGRG-3′) [19,20] are widely found within their promoter regions as shown in Fig. 1.

With the exception of the *celC* gene, no transcripts were detected in either the WT strain or the *AoxlnR* disruptant on Avicel and cellobiose. The *celC* gene was expressed even in the *AoxlnR* disruptant, indicating that transcription factor(s) different from AoXlnR also function in the inducible expression of the *celC* gene. Cellulose-inductive expression of the xylanolytic and cellulolytic genes observed with the *AoxlnR* overexpressed strain led to speculation that AoXlnR could mediate cellulose-inductive expression of the xylanolytic and cellulolytic genes in *A. oryzae.* However, caution is needed when dealing with artificially high-level expression of a transcription factor. Therefore, we tried to prove the involvement of endogenous AoXlnR in cellulose-dependent induction of the target genes by monitoring β-Gal activity in the WT strain transformed with an *E. coli lacZ* reporter gene under the control of the authentic and mutated *xynF1* promoter [8] (Fig. 4). The Avicel- and xylan-dependent expression of the gene was compared in each strain. β-Gal activity in all the strains was quite low, approximately 4 U/mg protein under the non-induced conditions. Under the Avicel-induced conditions, full inducibility was observed with transformants carrying the authentic promoter (WTZ), which was approximately four-fold lower than that of xylan-induced transformants. Mutation of high-affinity binding site (Mu1Z) decreased markedly the enzyme production, seven-fold lower than that

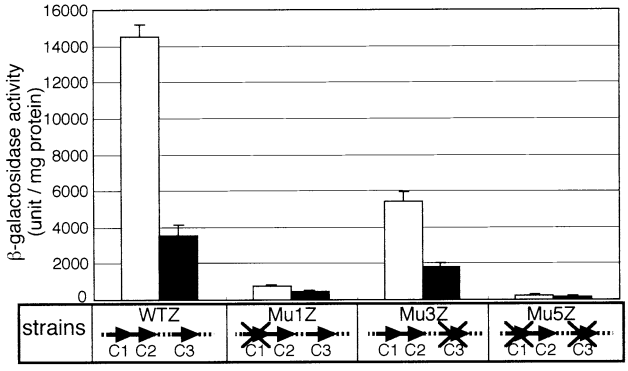

Fig. 4. β-Gal production in *A. oryzae* carrying an *E. coli lacZ* reporter gene under the control of the authentic or mutated *xynF1* promoter on Avicel and xylan. Each transformant grown for 24 h at 30°C in minimal medium supplemented with 1% polypeptone was collected by filtration, washed with minimum medium and transferred to minimal medium containing 1% xylan (white bars) or Avicel (black bars) as an inducing carbon source. After growth for an additional 48 h at 30°C, mycelial extracts were prepared for β-Gal assays as described under Section 2. The enzyme activity of three independent experiments is presented as the average values with standard errors (bars). Arrows represent the XlnR consensus binding sequences (C1; high-affinity binding sequence, C2; analogous sequence, C3; low-affinity binding sequence [8]). The arrows with **X** indicate mutated consensus binding sequences, where the second G essential for XlnR binding [2,8] was mutated to T.

of WTZ, whereas mutation of low-affinity binding site (Mu3Z) decreased the enzyme production only by two-fold compared to WTZ. Double mutation at both binding sites (Mu5Z) decreased most pronouncedly the enzyme production to 20-fold lower than that of WTZ. β-Gal was similarly expressed under the xylan-induced conditions. This clearly indicates that endogenous AoXlnR mediates the cellulose-inductive expression of the *xynF1* gene. Taken together with the cellulose-dependent induction of the genes observed with the *AoxlnR* overexpressed strain, AoXlnR can be concluded to mediate expression of the xylanolytic and cellulolytic genes in response to cellulose.

Recently, transcriptional factors, AceI and AceII, were isolated from *T. reesei* [21,22]. AceII is suggested to be involved in the cellulose-inductive expression of both the cellulase- and xylanase-encoding genes in *T. reesei* [22]. However, XlnR and AceII are different in size and show no significant amino-acid sequence similarity, indicating that the induction mechanism of the cellulolytic and xylanolytic genes in *T. reesei* could be different from that of aspergilli.

Major inductive signals for filamentous fungi to degradate plant cell wall are derived from cellulose and xylan. This is the first report to show that XlnR could receive inducing signals from these two different polysaccharides and activate transcription of the xylanolytic and cellulolytic genes in aspergilli. We are now trying to determine how AoXlnR recognizes two distinct inductive signals prior to induction of the target genes.

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