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Molecular Cloning of Xylanase Gene *xynG1* from *Aspergillus oryzae* KBN 616, a Shoyu Koji Mold, and Analysis of Its Expression

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A xylanase gene *xynG1* was isolated and sequenced from *Aspergillus oryzae* KBN616 used for making shoyu koji. The structural part of the *xynG1* gene was found to be 725 bp and was predicted to be interrupted by a single intron which was 62 bp in size. The mature XynG1 was predicted to be 189 amino acids in size and had high amino acid homology to other fungal xylanases classified in family 11 glycosidase. The XynG1 was detected in the culture supernatant of *A. oryzae* KBN616 grown in xylan medium but was not detected when *A. oryzae* KBN616 was grown in glucose medium. The *xynG1* gene was introduced into *A. nidulans* and was found to be expressed even in the glucose media. This expression pattern was confirmed using a luciferase gene as a reporter in *A. nidulans*.

[**Key words:** *Aspergillus oryzae*, gene expression, xylanase]

The filamentous fungus *Aspergillus oryzae* has been used in Japan for food production, for example, production of soy sauce or shoyu, miso and sake and has also been used for industrial enzyme production, for example, of α-amylase. *A. oryzae*, when grown on a shoyu koji composed of soybean and wheat, produces soybean cell wall-degrading enzymes. A greater efficiency of degradation of soybean cell wall would improve the usage of raw materials and decrease the amount of pressed cake left following the press-filtration of soy sauce mash which is difficult to dispose of. The major components of the plant cell wall are cellulose, lignocellulose, various hemicelluloses (*e.g.* xylan, arabinan, galactan and mannan) and pectin. Previously, we reported the cloning of the genes encoding a polygalacturonase and cellulases of *A. oryzae* KBN616 which is used for Japanese soy sauce production (1, 2). In these studies, we developed strains that produced a large amount of cellulase using genetic engineering.

Of the hemicelluloses mentioned above, the major compound is xylan. The main chain of this polysaccharide consists of β-1,4-linked D-xylopyranoside residues. The sugar residues can be partly modified by acetylation of xylose depending on the species from which the xylan originates. The main chain can be also branched as a result of arabinofuranose and glucuronic acid. Therefore, various kinds of enzymes are required for complete digestion of xylan. However, the cleavage of the backbone of β-1,4-linkage of xylose by endo-β-1,4-xylanase (1,4-β-D-xylan-xylanohydrolase EC 3.2.1.8), so designated xylanase, is most important for the degradation of xylan. Xylanase has broad potential biotechnological applications. At present, the majority of the commercial xylanolytic preparations are obtained from filamentous-fungus species. As with other xylanolytic microorganisms, filamentous fungi produce multiple xylanases whose genes have been previously cloned and sequenced. Although the cloning of many genes has been reported, only a few reports have been published regarding the mechanisms controlling fungal xylanase gene expression. The *A. tubigensis xlnA* gene has been shown to contain regulatory elements involved in xylan-specific induction and carbon catabolite repression within the promoter region (3). The regulation of *Chaetomium gracile cgxA* gene expression has also been conserved in *A. nidulans* (4). However, in case of *A. oryzae* which is a very important fungus in traditional fermentation industries and enzyme production, we do not have any information regarding its xylanases. As a first step toward characterizing the molecular properties of the *A. oryzae* xylanases and to elucidate their expression, we have cloned one of the xylanase genes of *A. oryzae* and analyzed its expression.

MATERIALS AND METHODS

Strains, plasmids and growth conditions *A. oryzae* KBN 616, a shoyu koji mold obtained from Bio'c (Toyohashi) was used to isolate DNA. *A. nidulans* G191 (*pyrG, pabaA1, fwA1, uaY9*) and plasmid pDJB1 (5) were generous gifts from Dr. G. Turner. Glucose or xylan medium (1% glucose or 1% oat spelt xylan, 0.5% yeast extract, 0.1% peptone, 0.1% K_2HPO_4, 0.05% $MgSO_4$ and 0.1% $NaNO_3$) was used for xylanase and luciferase expression by *A. nidulans* transformants. *Escherichia coli* DH5α was used for DNA manipulation.

Transformation of *A. nidulans* Transformations of *A. nidulans* G191 were performed as described by Ballance and Turner (5) with slight modifications. *A. nidulans* protoplasts were prepared from mycelial cells using Novozyme 234 (Novo). During the protoplasts preparation and transformation, 0.8 M NaCl was used as an osmotic stabilizer. Transformants of *A. nidulans* G191 were selected for uridine prototrophy.

Amplification of *xynG1* genomic DNA sequence by PCR and cloning of *xynG1* To amplify the *A. oryzae* xylanase gene using polymerase chain reaction (PCR), two oligonucleotide primers [5'-GA(AG)TA(TC)TA(TC)AT(TCA)GT(ATGC)GA(AG)(GA)A-3' and 5'-GCCCA(AG)AA(AG)TT(AG)AA(AG)TG(AG)TT-3'] were

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synthesized based on the amino acid sequences which are highly conserved among fungal xylanases. The amplified genomic DNA fragment was cloned to pUC19 and five independent inserts were sequenced. They comprised only one sequence and encoded 51 amino acids, the sequence of which was highly homologous to those of other fungal xylanases. The fragment was labelled with digoxigenin-11-dUTP (Boehringer Mannheim Biochemica) and used to screen the genomic library. A genomic library of *A. oryzae* KBN616 was constructed in Charomid 9-28 (Nippon Gene, Toyama) as described previously (1). Nucleotide sequences were determined using the dideoxy chain-termination method. All of the DNA manipulations were performed using standard methods as described by Sambrook *et al.* (6).

Measurement of enzyme activities Xylanase activity was assayed by the hydrolysis of Remazol brilliant blue-modified xylan (RBB-xylan; 5.75 mg/ml, Sigma, St. Louis, MO, USA) in 50 mM acetate buffer (pH 5.4) at 30°C. The reaction was terminated by the addition of 2 volumes of 96% ethanol. The increase in absorbance at 595 nm was used to express the xylanase activity as described by Biely *et al.* (7). One unit was defined as the increase in absorbance 1.0 per minute per ml culture filtrate. Protein was determined using the standard Bio-Rad protein assay with bovine serum albumin as a standard. For the luciferase assay, mycelia were collected by filtration with Whatman 3 MM filter. The mycelia were then ground in a lysis buffer and luciferase activity assayed using a Pica Gene Luminescence Kit (Nippon Gene). Luminescence was monitored by the Luminescence reader BLR301 (Aloka).

Antibody preparation and immunoblot analysis An antibody was raised against the recombinant XynG1 protein produced by *E. coli*. To create a DNA fragment which encodes the mature XynG1, an intron was removed using PCR with appropriate primers (Fig. 4A). The resultant fragment was fused downstream of the glutathione S transferase (GST) gene to *Bam*HI-*Sal*I in pGEX4T-2 vector (Pharmacia). The GST-XynG1 gene was expressed in *E. coli* and the fusion protein was purified using an affinity column. Then, the fusion protein was excised by thrombin and separated on SDS-PAGE (8). The acrylamide gel containing recombinant XynG1 was injected to a mouse to raise a specific antibody. Immunoblot analysis of the products was performed as described by Towbin *et al.* (9) after SDS-PAGE. To visualize proteins cross-reacted with the raised antibody to the XynG1, murine anti-IgG conjugated with horseradish peroxidase and the ECL-chemiluminascent system (Amersham) were used.

RESULTS AND DISCUSSION

Isolation and characterization of the *A. oryzae* xynG1 gene A genomic library of *A. oryzae* KBN616 constructed in Charomid 9-28 was screened with the 234 bp of PCR amplified DNA fragment as described above. Of 2×10^4 colonies screened, three positive clones were obtained. A 3.0-kb *Hin*dIII fragment of the insert was hybridized using the probe on Southern blotting analysis. This fragment was subcloned to pUC119 at *Hin*dIII site and designated as pXYNG1. A detailed restriction fragment map was constructed and sequencing was performed for both strands of *Xba*I-*Hin*dIII fragment as shown in Fig. 1. Although the exact translation-initiation site was not elucidated, the structural part of the *xynG1* appeared to be 725 bp long and was interrupted by a single intron of 62 bp (Fig. 2). The intron/exon junction which followed the GT-AG rule was present and the size of the intron resembled other filamentous fungal introns. Moreover, the intron occurs at the same position as those of other xylanases of filamentous fungi (3, 10–12). The *xynG1* contained an open reading frame encoding 221 amino-acid residues. Comparison of the deduced amino acid sequence of XynG1 with other fungal xylanases suggests that the precursor of XynG1 contains the signal sequence of 32 amino acids in its amino terminal. The secretory precursor is thought to be processed at a specific cleavage site between the Arg^{32} and Ala^{33} residues, resulting in the formation of a mature enzyme composed of 189 amino acids with a molecular weight of 20,602.

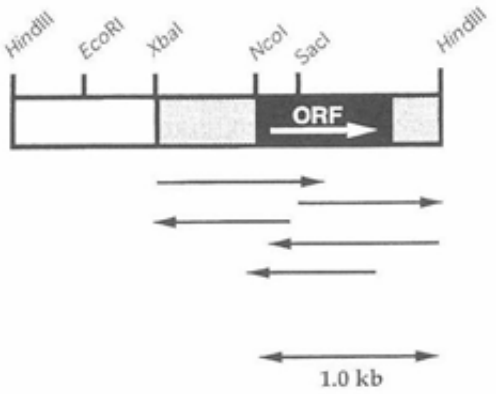

FIG. 1. Restriction map of the *xynG1* gene. The sequence strategy for the *xynG1* gene is indicated below the restriction map by arrows. The sequencing region is shaded. The open reading frame (ORF) is indicated by a black box with a white arrow which shows the direction of reading frame.

The 5′-noncoding region of the sequence was screened for various consensus sequences. Although no typical eukaryotic promoter with a TATA box (TATAAAT) existed, a similar sequence, TATAAA was detected 86 bp upstream from the translation initiation site. Furthermore, the CT rich sequence, which is considered as a fungal promoter element, was located just downstream of the TATA box-like sequence. Five sequences which are similar to the CREA-binding sites, 5′-G/CPyGGGG-3′ as were determined for *A. nidulans* (13), were found within the region upstream of the 873 bp fragment of *xynG1*. In common with some other fungal genes, the typical polyadenylation signal, AATAAA, was not present within the 3′-noncoding region. However, the tripartite terminator-like sequence which is thought to be implicated as polyadenlylation and termination signal in many filamentous fingi (14) was observed downstream of the ORF.

Comparison of the amino acid sequence of *A. oryzae* KBN616 XynG1 with other *Aspergillus* xylanases Xylanases can be grouped into two families, F and G, based on hydrophobic cluster analysis and sequence homology. In the numerical classification of glycosyl hydrolases, the families F and G correspond to families 10 and 11 (15). The deduced amino acid sequence of the mature XynG1 was compared to the published sequences for other family 11 xylanases from the *Aspergillus* species (Fig. 3). All of the xylanases in this group have β-

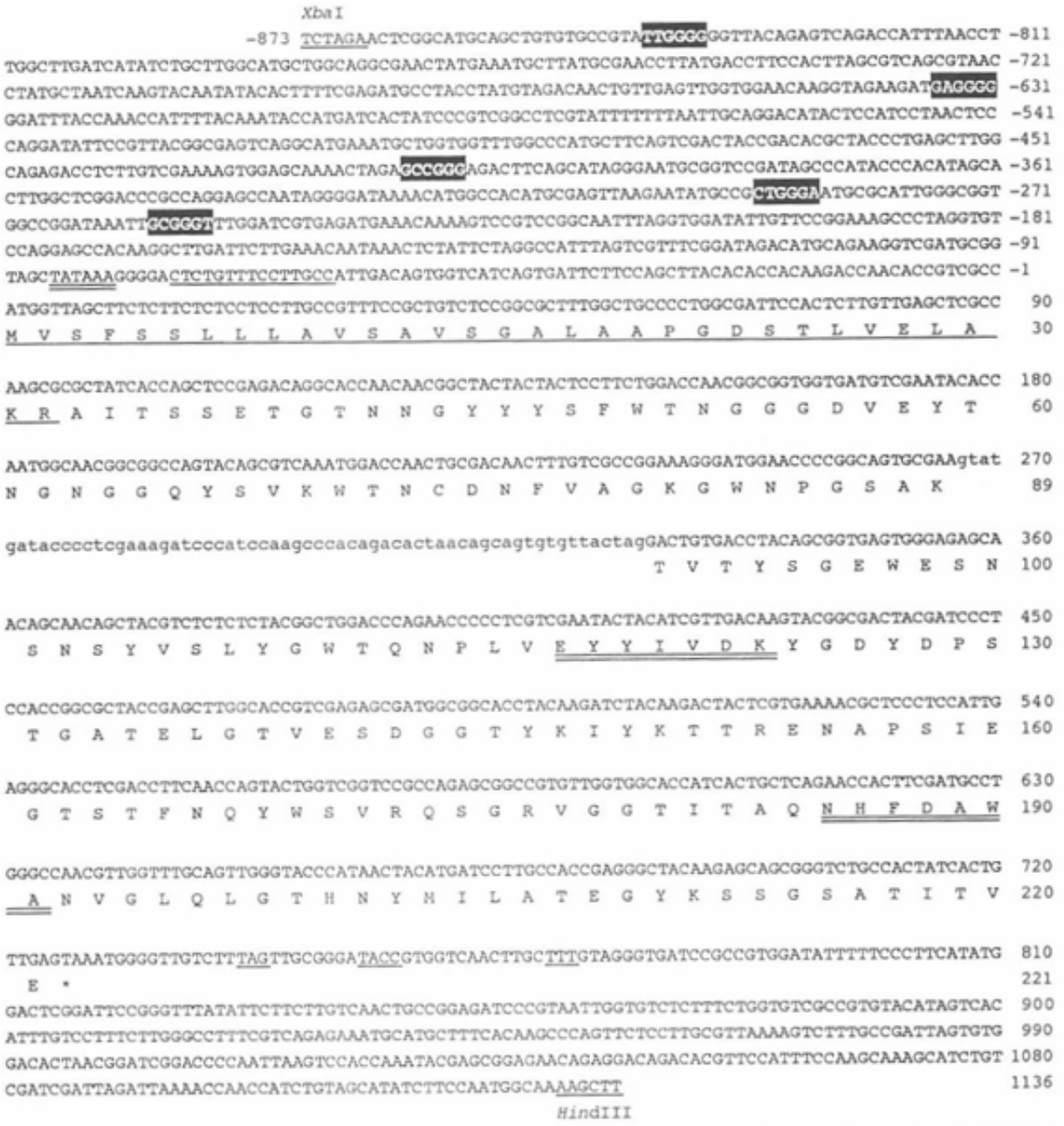

FIG. 2. Nucleotide and deduced amino acid sequences of *xynG1*. A putative intron sequence is in lower-case letters. Possible CREA-binding sites are boxed in black. A TATA-like sequence is double underlined and the CT-box is underlined. A termination sequence is underlined in the 3'untranslated region. A putative signal sequence for secretion is underlined. A double underlined amino acid sequence indicates the sequence used for the synthesis of PCR primers to amplify the *xynG1* gene fragment. This sequence has been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AB003085.

turns linking two β-sheet structures. An analysis of the secondary structures of XynG1 by the method of Chou and Fasman (16) also predicts that the polypeptide chain primarily forms β-sheets. Ko *et al.* reported that two glutamic residues of the *Bacillus pullulans* xylanase, which belongs to family 11 xylanase, were catalytic residues on the basis of their three-dimensional structure and site-directed mutagenesis (17). The two glutamic acid residues, Glu117 and Glu208, are also conserved in XynG1 (Fig. 3).

The deduced amino acid sequence of the mature *A. oryzae* KBN616 XynG1 revealed 65%, 43%, 63%, 65%, 63%, 44% and 44% homology to those of *A. kawachii* XynB (Genbank accession no. D38070), *A. kawachii* XynC (10), *A. nidulans* XlnA (11), *A. nidulans* XlnB (11), *A. niger* XynNB (12), *A. tubigensis* XlnA (3) and *A. awamori* xylanase (Genbank accession no. X78115), respectively. Among these xylanases, the enzymatic features of *A. kawachii* XynB, XynC (18) and *A. niger* XynNB (12) were reported. A crucial difference between

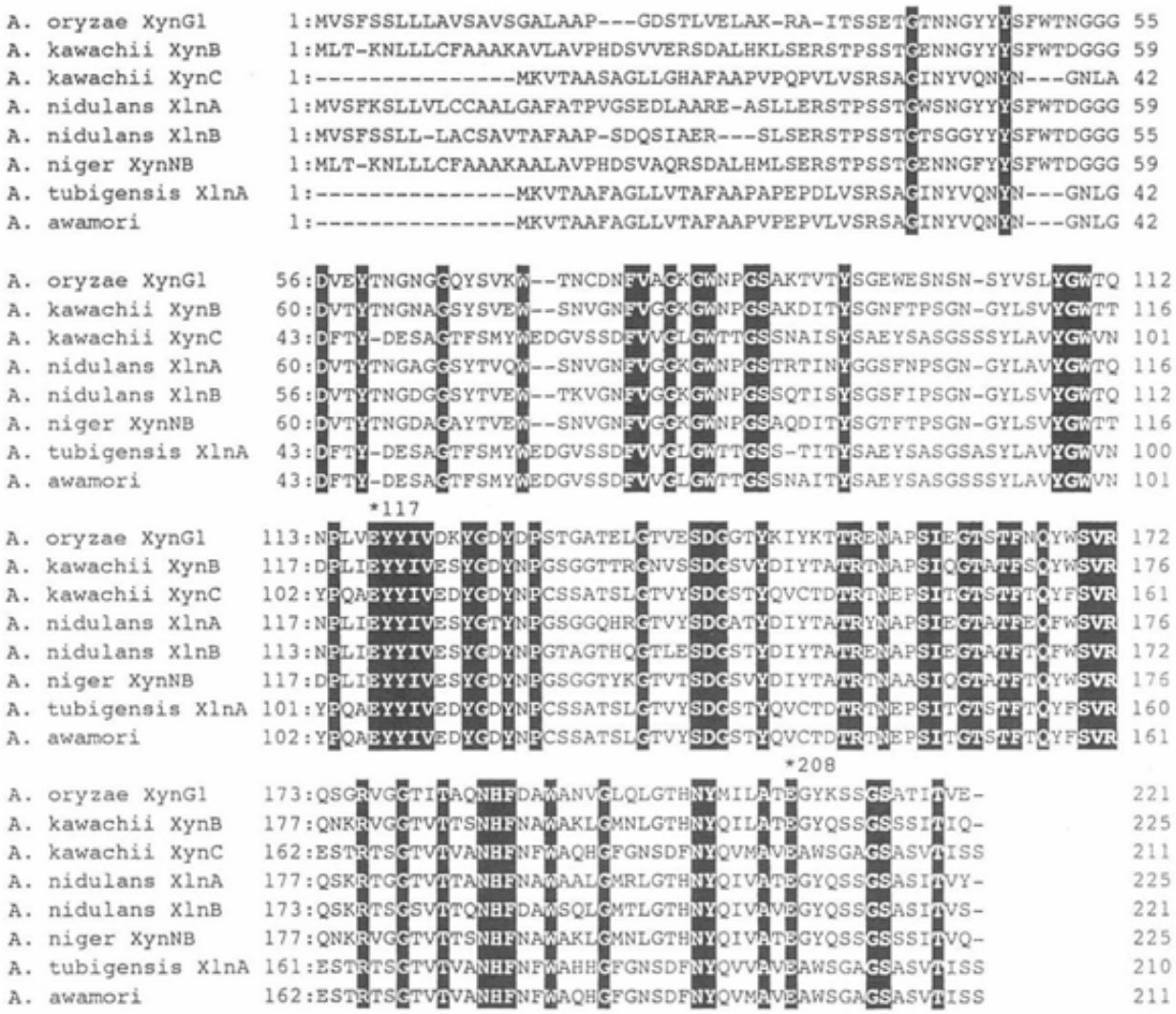

FIG. 3. Alignment of the amino acid sequences of the *A. oryzae* XynG1 with other *Aspergillus* xylanases. *A. kawachii* XynB (GenBank accession no. D38070), *A. kawachii* XynC (D14848), *A. nidulans* XlnA (Z49892), *A. nidulans* XlnB (Z49893), *A. niger* XynNB (D38071), *A. tubigensis* XlnA (L26988) and *A. awamori* (X78115). Sequences homologous to all eight sequences are boxed in black. The catalytic amino-acid residues are marked by asterisks.

them was their optimal pHs. *A. kawachii* XynB and *A. niger* XynNB exhibited weak acidophilic features with an optimal pH of 4.0. In contrast, *A. kawachii* XynC have acidophilic features with an optimal pH of 2.0. Based on the amino acid homologies of XynG1 with other xylanases, it may belong to the weak acidophilic group.

Expression of the *A. oryzae* xylanase gene The expression of *xynG1* in *A. oryzae* in xylan and glucose medium was analyzed using Western blotting analysis with anti-XynG1 antibody (Fig. 4). The antibody reacted with the polypeptide of 23 kDa present in the culture supernatant of the xylan medium. The molecular weight did not agree with the molecular weight 20,602 calculated using the deduced amino acid sequence. There are no possible N-glycosylation sites in XynG1. However, this discrepancy in molecular weight on SDS-PAGE may be the result of O-linked glycosylations at Thr or Ser residues. The results also indicate that *xynG1* is expressed in the presence of xylan as a carbon source and repressed in the presence of glucose. However, we cannot exclude the possibility that the antibody cross-reacted with another xylanase because the presence of more than two xylanases was reported in *A. nidulans* (18) and *A. kawachii* (19).

Although the repression/induction mechanisms of the fungal amylase genes were extensively studied, little is known regarding the mechanisms of the xylanase genes. Only the *A. tubigensis xlnA* gene was investigated, the regulatory element of which was found to be responsible for the expression and repression of the xylanase gene. Since de Graaff *et al.* reported that the regulation of xylanase induction was conserved in *A. tubigensis, A. niger* and *A. nidulans*, we have studied the functionality of the cloned *xynG1* gene by expressing it in *A. nidulans* (3). The *xynG1* gene (pAX1) was introduced into *A. nidulans* G191 by transformation using the *Neurospora crassa pyr*4 gene as a primary selection marker (Fig. 5). Five transformants were randomly selected and the copy number of the integrated *xynG1* in the genome was analyzed using genomic Southern blotting analysis where the

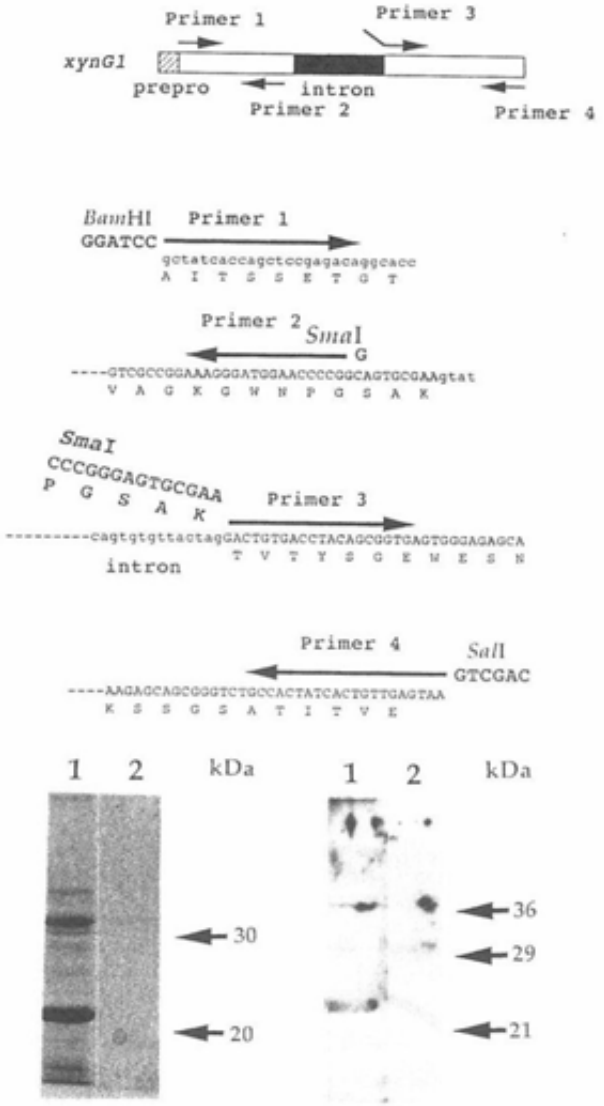

FIG. 4. Schematic diagrams of the synthesis of mature XynG encoding gene by PCR (A) and SDS-PAGE (left) and immunoblot (right) analysis of the xylanase secreted by *A. oryzae* KBN 616 (B). A: DNA fragment coding for mature XynG was synthesized by PCR with four primers. First and second exons were synthesized separately and subcloned into pGEX4T-2 at *Bam*HI-*Sal*I sites where first and second exons were ligated at newly introduced *Sma*I sites. B: Proteins in 1 ml of the culture filtrates of *A. oryzae* KBN616 grown in xylan (lane 1) and glucose (lane 2) medium for 3 d were precipitated with 10% trichloloacetic acid and applied to SDS-PAGE using 15% acrylamide gel. The same samples were used for immunoblot analysis.

Xba I digested genomic DNA from every transformant was separated on agarose gel and probed with labelled *xynG1* (Table 1). These transformants were then analyzed for gene expression after growth on oat spelt xylan or glucose medium as a carbon source. The expression was analyzed using the xylanase activity of culture filtrate (Table 1). All transformants had significant levels of xylanase activity on the glucose medium, whereas, no activity was detected in the culture supernatants of non-transformants on the glucose medium. The enzyme levels observed after growth on xylan medium were higher than those after growth on glucose. However, the activity of these transformants in xylan medium was comparable to that of non-transformants. This suggests that the expression level of *xynG1* is higher in glucose rather than in xylan medium. A simple explanation of this result is that D-xylan repressed the expression of *xynG1* in *A. nidulans*. Alternatively, repression by glucose was decreased because the concentration of repressor protein was diluted by the multiple integrated *xynG1* genes which bound to the repressor.

TABLE 1. Xylanase activities (unit/ml) of culture filtrates of several *A. nidulans* transformants carrying the *xynG1* gene

Carbon source	D-Glucose	D-Xylan	Copy number of the *xynG1* gene
A. nidulans transformants			
ANX#1	0.54	0.98	4–5
ANX#2	0.87	1.17	4–5
ANX#3	0.40	0.62	5–6
ANX#4	0.53	1.41	3–4
ANX#5	0.32	0.54	3–4
A. nidulans pDJB1	0.00	0.90	0
A. oryzae KBN616	0.36	15.1	1

We have developed a reporter system for the analysis of the upstream sequence of *xynG1* to enable further analysis of *xynG1* promoter in *A. nidulans*. This reporter system was based on the luciferase gene since the detection of luciferase activity is highly sensitive and there is no background activity in *A. nidulans* G191 cells. In this study, the putative *xynG1* upstream regulatory region was cloned in front of the luciferase ORF (Fig. 5). This plasmid was introduced into *A. nidulans* G191. After the selection of transformants, the expression of the reporter gene was studied in media containing D-glucose and xylan as a carbon source. Five transformants randomly selected exhibited luciferase activity both in glucose and xylan media (Fig. 6), whereas the non transformants did not show any activity in either media. Two transformants, no. 3 and no. 5, showed almost identical activities in glucose and xylan medium, that is, in these strains *xynG1* were expressed constitutively. Three other transformants, nos. 1, 2 and 4 exhibited much higher activity in glucose medium than in xylan medium. Southern hybridization analysis of the *Sac*I-*Eco*RI digested genomic DNA of the transformants revealed that all transformants contained several copies of the complete *xynG1* promoter-luciferase fusion gene, suggesting that luciferase expression was driven by a *xynG1* promoter sequence. Luciferase activities of all transformants except no. 3 in glucose media roughly corresponded to the relative copy number of the integrated luciferase gene. Differences in luciferase activities may also be explained by differences in the position at which the plasmid was integrated.

de Graaff *et al.* reported that xylose and arabinose were inducers for xylanase expression in several *Aspergillus* species (3). However, when *A. oryzae* KBN616 was cultivated with xylose as a carbon source, we did not observe any xylanase activity (data not shown). Therefore, the expression mechanism of the xylanase gene of *A. oryzae* KBN616 is different from other *Aspergillus* strains. It may be concluded that industrial strains such as *A. oryzae* KBN616 have several mutations concerning the expression of extracellular enzymes because they are mutagenized during breeding. The promoter analysis

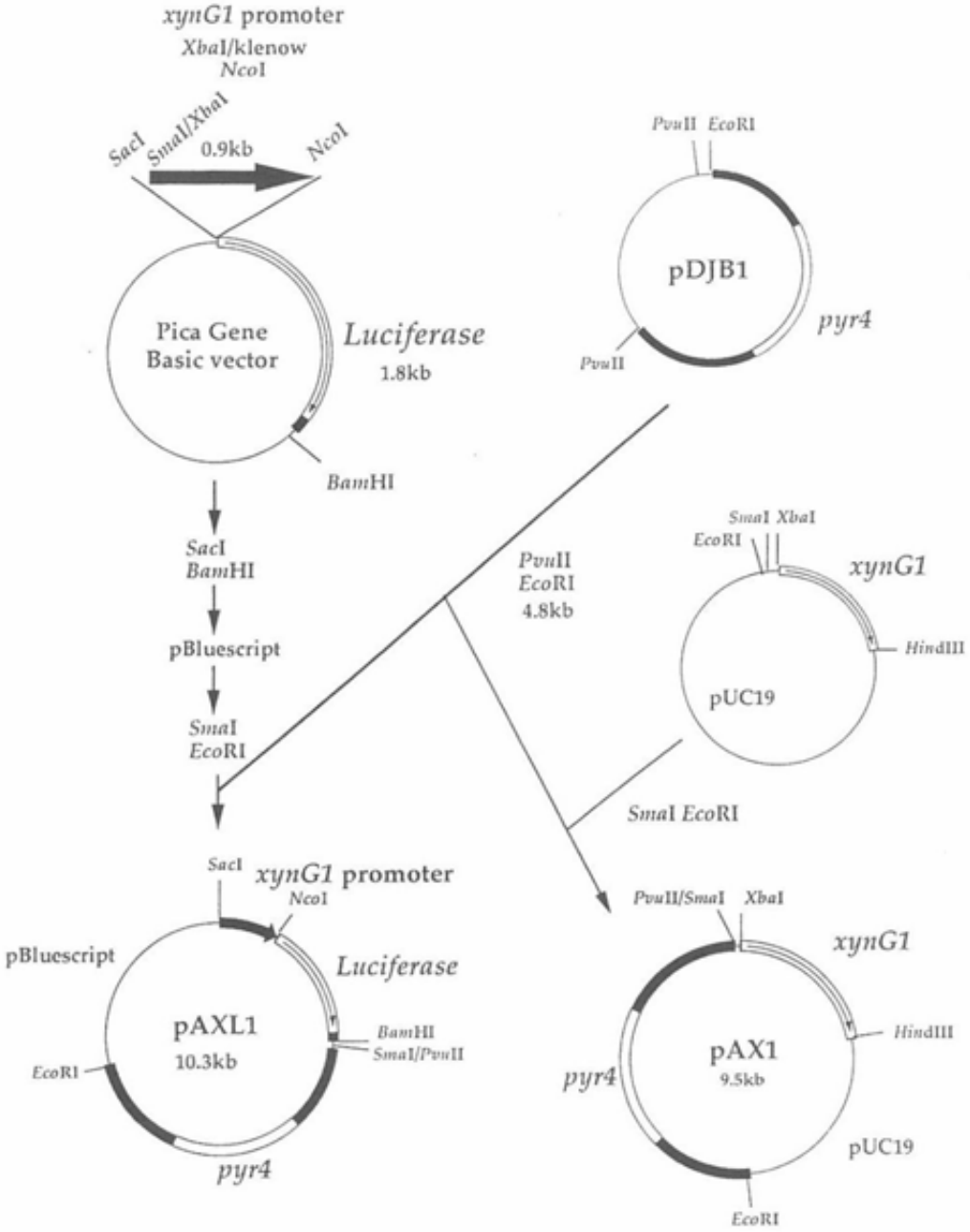

FIG. 5. Construction of expression plasmids for *xynG1* and reporter gene. To determine whether the *xynG1* could be expressed in *A. nidulans*, plasmid pAX1 was constructed. For the construction of pAX1, 2-kb of *Xba*I-*Hin*dIII fragment shown in Fig. 1 was subcloned to the *Xba*I-*Hin*dIII sites on pUC19, followed by digestion with *Sma*I and *Eco*RI. *Eco*RI-*Pvu*II fragment containing *pyr4* from pDJB1 was subcloned into *Sma*I-*Eco*RI sites of the above plasmid. For the construction of pAXL1, *Xba*I-*Nco*I fragment of *xynG1* which was the upstream region of *xynG1* ORF was cloned between the *Sma*I-*Nco*I sites of Pica Gene basic vector 2 (Nippon Gene, Toyama), followed by digestion with *Sac*I-*Bam*HI which generated an approximately 2.7-kb DNA fragment containing *xynG1* promoter-luciferase fusion gene. This fragment was subcloned into *Sac*I-*Bam*HI sites in pBluescript II KS+ vector, followed by digestion with *Sma*I and *Eco*RI, to which the *Eco*RI-*Pvu*II fragment of *pyr4* from pDJB1 was subcloned.

using the reporter gene suggested this hypothesis. However, we cannot exclude the possibility that the sequence responsible for catabolite repression is located further upstream when considering the difference between the luciferase expression patterns of the transformants. Putative CREA-binding sequences were observed in more than 600 bp upstream of *Trichoderma konignii* CBHI gene (20).

In this study, we cloned the *xynG1* gene which encodes the low molecular weight xylanase of *A. oryzae* KBN616, belonging to glycosidase family 11. Western blotting analysis suggests that expression of *xynG1* was induced by xylan and repressed by glucose in *A. oryzae* KBN616. However, the expression of *xynG1* was not

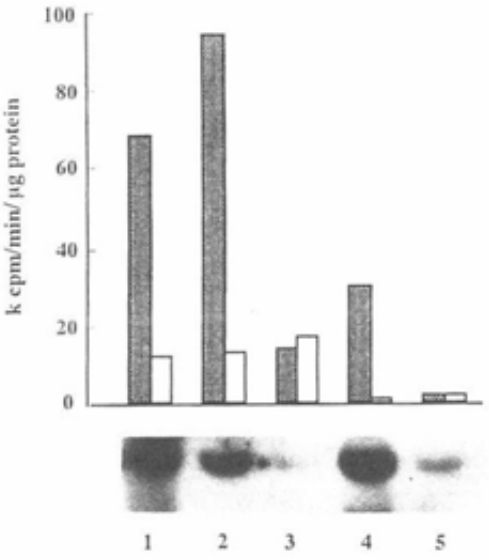

FIG. 6. Luciferase activity in *A. nidulans* transformants carrying the pAXL1 plasmid. Transformants were cultured in medium containing glucose (shaded bar) or xylan (white bar) and luciferase activity was monitored as described in Materials and Methods. Relative copy numbers of integrated *xynG1* promoter-luciferase fusion gene were analyzed using genomic Southern blotting with the luciferase gene as a probe. Ten μg of total DNA from the transformants were digested with *Sac*I and *Bam*HI and used for Southern hybridization.

repressed by glucose in *A. nidulans* G191. This was also confirmed by reporter gene analysis. Further analysis is required to determine xylanase gene expression in industrial *A. oryzae* strain and to allow us to breed a strain which efficiently produces plant cell wall-degrading enzymes.

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