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Marco M.C. Gielkens · Jaap Visser · Leo H. de Graaff

Arabinoxylan degradation by fungi: characterization of the arabinoxylan-arabinofuranohydrolase encoding genes from *Aspergillus niger* and *Aspergillus tubingensis*

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Abstract The genes encoding the enzyme arabinoxylan arabinofuranohydrolase, which releases L-arabinose from arabinoxylan, have been cloned from the closely related fungi *Aspergillus niger* and *Aspergillus tubingensis* and were shown to be functional in *A. niger*. Integration of multiple copies in the genome resulted in over-expression of the enzymes. The arabinofuranohydrolases encoded comprise 332 amino acids and have 94% amino acid identity. Their primary structure is not related to those of other α-L-arabinofuranosidases, except for a low similarity with XYLC, a bacterial α-L-arabinofuranosidase from *Pseudomonas fluorescens* which acts on oat spelt xylan. The *axhA* expression pattern in *A. niger* differed from that of *abfB*, since it was strongly induced by birchwood xylan and much less by L-arabitol or L-arabinose. Furthermore, Northern analysis revealed that *axhA* expression was de repressed in *creA*^{d} mutants and carbon catabolite repressed by D-glucose.

Key words Aspergillus · Arabinofuranohydrolase · Gene structure · Regulation · *axhA* expression · Arabinoxylan

Introduction

Microbial degradation of structural polysaccharides of the plant cell wall, such as cellulose, hemicellulose and pectin, is an important process in nature. A wide variety of enzyme activities, mainly produced by saprophytic fungi and bacteria, are involved in this degradation process. Of the hemicelluloses known, xylan is the most abundant compound. The main chain of this polysaccharide consists of β-1,4-linked D-xylopyranoside residues. Depending on the species from which the xylan originates, the sugar residues can be partly modified by acetylation of D-xylose at the O-2 or the O-3 position as is the case, for instance, in xylan from birch wood. The main chain can also be branched as a result of O-2- or O-3-linked L-arabinofuranose and O-2-linked D-glucuronic acid and 4-O-methyl-D-glucuronic acid residues (Wilkie 1979).

Due to the complex composition of xylan, a spectrum of enzyme activities is necessary for the complete hydrolysis of this polysaccharide. Endo-xylanases (E.C. 3.2.1.8) are only capable of hydrolysing the xylan backbone at non-modified residues. Therefore microorganisms synthesize a number of enzymes capable of removing substituents from the xylan backbone, e.g. α-L-arabinofuranosidase B (ABFB)(E.C. 3.2.1.55) (van der Veen et al. 1991), acetyl xylan esterase (AXEA) (E.C. 3.1.1.6) (Kormelink et al. 1993 b) and (1,4)-β-D-arabinoxylan arabinofuranohydrolase (AXHA) (Kormelink et al. 1991). These enzymes, which were all identified in various *Aspergillus* species, show strong synergistic effects with endo-xylanase activity resulting in an enhanced degradation of xylan.

AXH was first isolated and described by Kormelink et al. (1991). This enzyme releases 1,2-α- and 1,3-α-linked arabinofuranosyl groups from arabinoxylans and arabinoxylan-derived oligosaccharides but not from other arabinose-containing substrates such as arabinans and arabinogalactans (Kormelink et al. 1991, 1993 a). This is in contrast to ABFB which also shows arabinose-releasing activity towards L-arabinan and arabinogalactan and 1,5-α-linked α-arabinofuranosyl oligosaccharides (van der Veen et al. 1991). From kinetic experiments it was concluded that AXH is able to release arabinofuranosyl substituents from terminal, as well as from non-terminal, single substituted xylopyr-

M.M.C. Gielkens · J. Visser · L.H. de Graaff (✉)
Section of Molecular Genetics of Industrial Microorganisms, Wageningen Agricultural University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands

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anosyl residues in low molecular weight oligosaccharides, whereas ABFB can only release arabinofuranosyl substituents from terminal single substituted xylopyranosyl residues (Kormelink et al. 1993 a).

In the present study we have cloned the *axhA* gene from two related *Aspergilli*. Kusters-van Someren et al. (1991 b) proposed that the *A. niger* aggregate consists of two distinct species, *A. niger* and *A. tubingensis*. Although these two species are very closely related, the divergence between them has consequences for the properties of some cell-wall degrading enzymes and their industrial application. Qualitative differences were found in the xylanase spectrum of both species; i.e. the *xlnA* gene is present in the *A. tubingensis* but absent in the *A. niger* genome (de Graaff et al. 1994). Differences can also be quantitative, as was reported by Bussink et al. (1991) for the polygalacturonase encoding *pgaII* genes of both strains. In view of this, it is useful to isolate and compare the *axhA* gene of both species.

Materials and methods

Fungal strains, bacterial strains, phages and plasmids. A. tubingensis NW756 (Kusters-van Someren et al. 1991 b) and *A. niger* N402 (*csp*A1), which is a low-conidiophore derivative from N400 (CBS 120.49), were used as wild-type strains in all the experiments described. In *A. niger* transformation experiments the N402-derived strain NW219 (*cspA1 leuA1 nicA1 pyrA6*) was used as a recipient. Strains NW138 (*cspA1 fwnA6 nicA1 pacC2*), 502.17 (*cspA1 creA*^{d}2) and 555.6 (*cspA1 creA*^{d}4) were used in transfer experiments to analyze *axhA* expression. *Escherichia coli* strains DH5α (Gibco-BRL, Gaithersburg M., USA) LE392, and XL1-Blue MRF′ were supplied by Stratagene. Phage ExAssist (Stratagene Cloning Systems, La Jolla, Calif., USA) was used as a helper phage for phagemid excision. Plasmid vectors pBluescript II SK^{-} (Short et al. 1988), pUC19 (Yanisch-Perron et al. 1985), pGEM5, pGEM7 and pGEM-T (Promega, Madison, Wis., USA) were used for subcloning. Plasmid pGW635, which contains the *A. niger pyrA* gene encoding orotidine-5′-phosphate decarboxylase, was used to co-transform *A. niger* (Kusters-van Someren et al. 1991 a).

Aspergillus cultivation: media and conditions. All the media used for fungal growth and induction experiments were based on *Aspergillus* minimal medium, which had the composition described by Pontecorvo et al. (1953). The final pH was adjusted to 6.0. Carbon sources were added to the minimal medium as indicated in the particular experiments. The growth temperature was 30 °C in all cases. The mycelium to be used for protoplast formation was pregrown for 18 h on minimal medium with 100 mM of D-glucose as a carbon source supplemented with 0.2% casamino acids and 0.5% yeast extract. Where necessary the media were supplemented with 10 μM nicotinamide, 2 mM leucine or 10 mM uridine.

Amino acid sequence determination, cyanogen bromide cleavage and enzymatic deglycosylation of A. tubingensis AXHA. Approximately 1 nmol of purified AXHA was subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P (Millipore, Bedford, Mass., USA) polyvinylidene difluoride membranes (Matsudaira 1987). The membrane fragment containing the main band, with an apparent molecular mass of 32 kDa, was employed for sequence analysis using a gas-phase sequencer equipped with a PTH analyzer as described by Amons (1987). In addition, the sequence of an internal peptide was also determined. For this, approximately 2 nmol of purified AXHA was cleaved using a 200-fold molar excess of CNBr. The resulting peptides were separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto an Immobilon-P membrane. The appropriate piece of membrane containing a peptide with an apparent molecular mass of 9 kDa was recovered and used in sequence analysis. N-linked carbohydrate moieties were removed from purified, denatured AXHA by treatment with N-glycanase F (Boehringer Mannheim, Germany) as recommended by the manufacturer.

Construction and screening of an arabinoxylan-induced cDNA library of A. niger N400. A. niger N400 was cultivated for 69 and 81 h using 2% wheat arabinoxylan as a carbon source. Total RNA was isolated by using the guanidium thiocyanate/CsCl protocol described in Sambrook et al. (1989), except that the RNA was centrifuged twice using a CsCl gradient. Poly A^{+} mRNA was isolated from 5 mg of total RNA by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972; Sambrook et al. 1989) with the following modification; SDS was omitted from all solutions. PolyA^{+} RNA was pooled and 7 μg was used to synthesize cDNA. 120 ng of cDNA were ligated into 1.2 μg of bacteriophage lambda λ Uni-ZAP XR vector arms using the ZAP-cDNA synthesis kit (Stratagene) according to the manufacturer's instructions. After ligation of the cDNA into Uni-ZAP XR vector arms, the phage DNA was packaged using Packagene extracts (Promega) according to the manufacturer's instructions resulting in a primary library consisting of 3.5×10^{4} independent recombinant clones. The primary library was amplified using *E. coli* XL1-Blue MRF′, titrated, and stored at 4°C. The immunochemical screening of the cDNA expression library was basically performed as described by Flipphi et al. (1993 b) using anti-AXHA antiserum raised against *A. tubingensis* AXHA in a New Zealand white rabbit.

Polymerase chain reaction (*PCR*). The amino acid sequence of the internal peptide fragment was used to derive the oligonucleotide mixture AB4264 (5′-ATG ATK GTI GAR GCI ATK GG-3′), in which I stands for an inosine, K for an A, T or C, and R for an A or G. This oligonucleotide mixture was used in PCR in combination with the T7 sequence primer (Stratagene). As a template for amplification, 50 ng of λ-cDNA, isolated from the cDNA library phage stock according to Sambrook et al. (1989), was amplified using the following sequence: the DNA was heat-denatured by incubation for 3 min at 95°C which was followed by 25 cycles of 1 min at 95°C, 1 min at 42°C and 1 min at 72°C. The reaction was terminated after a final 5-min incubation at 72°C.

Isolation and cloning of the A. niger and A. tubingensis axhA genes. The *A. tubingensis* genomic library in λEMBL3 (de Graaff et al. 1994) was screened by using a 500 bp fragment, generated by PCR and containing *A. niger axhA* cDNA sequences, as a probe. The *A. niger* N400 genomic library in λEMBL4 (Harmsen et al. 1990) was screened using a 1.2 kb *Eco*RI-*Xho*I fragment from a positive cDNA clone (see Results). In both cases approximately 1.5×10^{4} pfu were screened using the following conditions for hybridization. After pre-hybridization of the filters for 2 h in pre-hybridization buffer containing 6 × SSC (Sambrook et al. 1989), ^{32}P-labelled probe was added to the pre-hybridization solution and hybridized overnight at 65°C. After hybridization the filters were washed down to 0.1×SSC at 65°C. Other DNA manipulations, such as plasmid DNA isolation, lambda DNA isolation, Southern blot analysis and subcloning, were performed as described by Sambrook et al. 1989.

DNA sequence determination, sequence analysis and primer extension mapping. For sequencing, DNA fragments were subcloned into the plasmid vectors pBluescript, pGEM and pUC. The DNA

sequence was determined by the dideoxynucleotide chain-termination method (Sanger et al. 1977) using the T7 Sequencing Kit from Pharmacia LKB Biotechnology according to the supplier's instructions. Alkali-denatured plasmid DNA was used as template and both universal oligonucleotides as well as gene-specific oligonucleotides were employed as primers. Computer analysis was done using the PC/GENE programme (Intelligenetics) and the University of Wisconsin software (Devereux et al. 1984). Primer extension mapping to determine transcription initiation sites was performed according to Calzone et al. (1987) using polyA$^+$ RNA isolated from transformants containing multiple copies of either the *A. niger* or the *A. tubingensis axhA* gene. These strains were grown for 40 h on minimal medium containing 1.5% (w/v) wheat arabinoxylan as a carbon source. RNA was isolated from mycelial powder with TRIZol (Gibco-BRL) according to the manufacturer's instructions. PolyA$^+$ RNA was isolated from 2 mg of total RNA as described above.

Northern analysis. Mycelium to be used in transfer experiments was pre-grown for 18 h on minimal medium with 100 mM of D-fructose as a carbon source, supplemented with 0.2% casamino acids and 0.5% yeast extract. After harvesting and washing with saline, 3 g of mycelium (wet weight) were transferred to 250-ml Erlenmeyer flasks containing 50 ml of minimal medium and grown for an additional 6–12 h. Total RNA was isolated with TRIZol (Gibco-BRL) according to the manufacturer's instructions and 20 µg of total RNA was loaded on formaldehyde-agarose gels (Sambrook et al. 1989), transferred to Hybond-N membranes (Amersham) and UV-cross linked. Hybridization and washing of the membranes were carried out essentially as described by Sambrook et al. (1989).

Analysis of culture-medium protein. After growth of the fungus, the mycelium was removed by filtration over a Büchner funnel. The resulting culture medium samples were then centrifuged (8000 g, 10 min) to pellet debris. Proteins secreted into the medium were analyzed by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS according to Laemmli (1970) on a midget gel-electrophoresis system (Pharmacia LKB). For the specific detection of AXHA, Western analysis was used by incubating nitrocellulose blots with anti-AXHA antiserum followed by staining with alkaline phosphatase-labelled goat anti-rabbit IgG conducted as described by the manufacturer (Bio-Rad, Richmond, Calif., USA).

Results

Determination of amino acid sequences

The AXHA enzyme was purified from medium filtrates after culturing *A. tubingensis* on crude wheat arabinoxylan. These filtrates, which were enriched in AXHA, were a kind gift of Gist-brocades. The apparent molecular mass of the purified enzyme, as determined by SDS-PAGE using a 10% gel, was 32 kDa and the isoelectric point was determined to be 3.6. These data are in agreement with those reported for AXH by Kormelink et al. (1991). This enzyme was also used to raise antibodies. These antibodies also reacted strongly with *A. niger* AXHA.

N-terminal amino acid sequences were determined for the isolated mature protein as well as for a 9 kDa peptide obtained by cyanogen bromide cleavage. The N-terminal amino acid sequence of the whole protein was determined to be [K X A L P S S Y]. The amino acid residue at position 2 could not be assigned and is therefore designated as "X". Since in the sequencing procedure cysteine residues are only determined if the protein is *S*-pyridylethylated before analysis, which is not the case here, it is possible that a cysteine occurs at this position. For the internal cyanogen bromide fragment the N-terminal amino acid sequence [I V E A I G S T G H R Y F (R/N) (S) (F) (T)] was found. As the last four amino acids are ambiguous, these are given between brackets.

Isolation of an *A. niger axhA* cDNA clone

The wild-type strain N402 was grown on minimal medium containing 2% (w/v) crude wheat arabinoxylan, and samples of the culture medium were taken 48, 69, 81 and 96 h after inoculation. Analysis of these samples by Western blot analysis for AXHA expression revealed high expression levels of the enzyme at 69, 81 and 96 h after inoculation (data not shown). Mycelia harvested at 69 and 81 h were chosen to construct a cDNA expression library. After amplification of the library, 5×10^4 pfu were immunologically screened for expression of the AXHA cDNA. About 50 positive plaques were found. Upon purification and excision of eight positive clones, the resulting plasmids were isolated and cDNA insert lengths were determined by digestion with *Eco*RI and *Xho*I and subsequent agarose electrophoresis. All eight clones were partially sequenced at both the 5′ and the 3′ ends of the cDNA. All of them contained the complete coding region, since the N-terminal amino acid sequence, as determined for the mature AXHA protein, was in all cases confirmed by the nucleotide sequence. cDNA clone pC61A had the longest cDNA insert, as it contained a 5′ leader sequence of 56 nucleotides in front of the putative translation start site. The complete nucleotide sequence of this clone was determined for both strands.

Isolation of the *A. niger axhA* gene

To obtain the AXHA encoding gene, the *A. niger* N400 genomic library (Harmsen et al. 1990) was screened using a 1.2 kb *Eco*RI-*Xho*I fragment of cDNA clone pC61A. This resulted in the isolation of ten positive clones. The inserts of four of these phages were partially characterized by Southern analysis. In all four clones, fragments originating from the same genomic region were found and a 3.7 kb *Xho*I fragment containing the AXHA encoding gene was cloned into pBluescript SK$^-$, resulting in pIM3002 (Fig. 1).

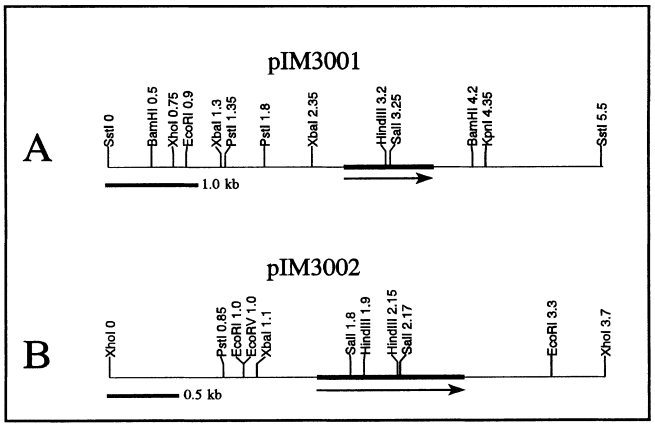

Fig. 1 **A** restriction map of the 5.5 kb *Sst*I fragment, present on plasmid pIM3001, containing the *A. tubingensis axhA* gene and its flanking regions. The position of *axhA* is represented by the *black bar*, the *arrow* indicates the direction of transcription. **B** restriction map of the 3.7 kb *Xho*I fragment, present on the plasmid pIM3002, containing the *A. niger axhA* gene and its flanking regions. The position of *axhA* is represented by the *black bar*, the *arrow* indicates the direction of transcription

Generating a probe for the *A. tubingensis* AXHA encoding gene and isolation of the *A. tubingensis axhA* gene

The partial amino acid sequence of the internal CNBr fragment was used to design the complex oligonucleotide mixture AB4264. This oligonucleotide mixture was derived from amino acid 1 (I) to amino acid 6 (G) including the methionine residue of the peptide preceding the CNBr fragment.

The oligonucleotide mixture was used in combination with the universal sequence primer T7 in a PCR on λ-cDNA isolated from the cDNA library described above. Analysis of the reaction products revealed two distinct products of about 500 bp and 600 bp. Both PCR fragments were cloned into pGEM-T and partially sequenced for identification. The 500-bp PCR fragment had a high degree of identity with the nucleotide sequence of the *A. niger* cDNA clone and included also the amino acid sequence of 17 residues of the internal cyanogen-bromide fragment. The nucleotide sequence of the first 220 bp of the 600-bp PCR fragment showed significant homology with aldose/aldehyde reductases from several organisms and was therefore regarded as a PCR artefact.

Using the 500 bp PCR fragment described above, a *A. tubingensis* genomic library (de Graaff et al. 1994) was screened by homologous hybridization, to isolate the gene encoding AXHA resulting in three hybridizing plaques. The inserts of the three phages were characterized by limited Southern analysis. In all three clones, fragments originating from the same genomic region were found and a 5.5 kb *Sst*I fragment containing the AXHA-encoding gene from *A. tubingensis* was cloned into pBluescript SK^-, resulting in pIM3001 (Fig. 1).

Primary structure of the *A. niger* and *A. tubingensis axhA* genes and their deduced protein structure

The nucleotide sequences of both the *A. niger axhA* (pIM3002) and the *A. tubingensis axhA* (pIM3001) genes were determined for both strands by subcloning fragments from pIM3001 and pIM3002, in combination with the use of specific oligonucleotides as primers in the sequencing reactions. The sequence determined for the *A. niger axhA* gene was 2098 bp long and contained 780 bp of the 5′ non-coding region and 322 bp of the 3′ non-coding region. The sequence determined for the *A. tubingensis axhA* gene was 2177 bp long and contained 823 bp of the 5′ non-coding region and 358 bp of the 3′ non-coding region. The structure of the two *axhA* genes was very similar and the nucleotide sequences were 82% identical. The nucleotide sequence identity in the coding region, viz. 89%, was higher than in the 5′ and 3′ non-coding flanking sequences (80% and 70%, respectively).

The transcription start points (*tsp*) were determined by primer extension mapping using $polyA^+$ RNA isolated from transformants containing multiple copies of either the *A. niger* or the *A. tubingensis axhA*. Both strains were grown for 40 h on minimal medium containing 1.5% (w/v) crude wheat arabinoxylan as a carbon source. For both genes a major *tsp* was found at position -64 and two minor *tsp*s were found at positions -57 and -71 relative to the translation start site. The leader of the longest *A. niger* cDNA clone (pC61A) starts one nucleotide downstream from the minor *tsp* at position -57. Analysis of the 5′ non-coding region of the *A. tubingensis axhA* gene revealed the presence of sequence elements resembling general promoter elements for fungal genes (Gurr et al. 1987; Unkles 1992), such as a TATA and a CAAT box, located respectively 46 bp and 108 bp upstream of the major *tsp*. In the 5′ non-coding region of the *A. niger axhA* gene, the elements AAATAT and CTAAT were found at corresponding positions. In addition, a CT-stretch directly upstream of the major *tsp* was found in the promoter of both genes.

Analysis of the 3′ end of the *A. niger axhA* cDNA clones revealed two transcription stops, 172 and 183 bp downstream from the stop codon. Although a few AT-rich sequences were found in the 3′ non-coding region of both genes, the consensus polyadenylation signal, which is sometimes found in other fungal genes, was not present (Proudfoot 1991).

The coding regions of both genes consisted of a single open reading frame of 996 bp, and contained no introns. This was concluded by comparing the *A. niger* cDNA and genomic *axhA* sequences. The open reading frame encodes a protein of 332 amino acid residues. The

derived amino acid sequences for both genes were 94% identical. The N-terminal amino acid sequence, as determined for the mature *A. tubingensis* AXHA, is preceded by a pre-sequence of 26 residues, which presumably serves as a signal peptide. The cleavage site between residues 26 and 27 conforms to the "-3 -1" rule with a small, non-aromatic/charged amino acid (valine) at the -3 position and a small amino acid (alanine) at the -1 position, as proposed by von Heijne (1986) for the cleavage of signal peptides. The amino acid sequence of the internal fragment was found to be present from position 233 to 249 in the derived amino acid sequence. Removal of the signal sequences leaves a mature protein of 306 amino acid residues in both cases, which have deduced molecular mass values of 33250 Da and 33101 Da and a theoretical isoelectric point of 4.2 and 4.1 for *A. tubingensis* AXHA and *A. niger* AXHA, respectively. Furthermore, the *A. tubingensis* AXHA contains one possible N-glycosylation site at position 313 whereas the *A. niger* AXHA does not. However, the difference in apparent molecular mass, as observed in Fig. 2 A, was not resolved by enzymatic N-deglycosylation with N-glycanase F. The possibility of O-glycosylation was not examined.

Expression of the *A. niger* and *A. tubingensis axhA* genes in *A. niger* grown on wheat arabinoxylan

To investigate whether the cloned genes were functional, the plasmids pIM3001 and pIM3002 were introduced in *A. niger* NW219 by co-transformation using pGW635, which carries the *A. niger pyrA* gene, as the primary selection marker. Nineteen *A. niger* prototrophic transformants were randomly chosen in each case and analyzed for the expression of AXHA. The transformants and the *A. niger* N402 control strain were grown on minimal medium containing crude wheat arabinoxylan. Samples of the growth media were taken at 20 and 41 h after inoculation for the transformants of the *A. tubingensis axhA* gene and at 24 and 40 h for the transformants of the *A. niger axhA* gene. Culture filtrates were analyzed by Western analysis using anti-AXHA antibodies. Twelve of the nineteen transformants of the *A. tubingensis axhA* gene and eight of the nineteen transformants of the *A. niger axhA* gene analyzed over expressed the corresponding gene product (Fig. 2). Southern analysis of transformants of the *A. niger axhA* gene confirmed that the highly overproducing strains contained multiple copies (5–10) of the *A. niger axhA* gene, integrated in tandem.

Induction and carbon catabolite repression of AXHA expression in *A. niger*

The expression of both AXHA and ABFB, encoded by *abfB* (Flipphi et al. 1993 a), was studied in transfer experiments. *A. niger* wild-type strain N402 was pregrown on 2% D-fructose and aliquots of mycelium were transferred to minimal medium containing either L-arabinose, L-arabitol, D-fructose, D-glucose, D-xylose (all 50 mM) or 1% (w/v) birchwood xylan. These cultures were grown for additional growth periods of 6 h and 12 h. Western analysis of the culture filtrates revealed that AXHA was mainly expressed when grown on xylan and much less on L-arabitol, whereas ABFB was highly expressed on almost all carbon sources except D-glucose and D-fructose (Fig. 3, panels a and c). Northern analysis of *axhA* transcription showed similar

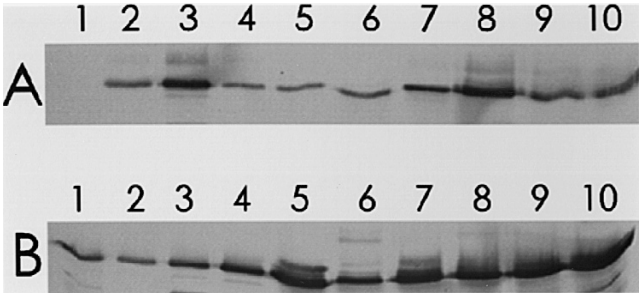

Fig. 2 A,B Western-blot analysis of AXHA expression by *A. niger* wild-type N402 and *A. niger* transformants upon growth on minimal medium containing 1.5% crude wheat arabinoxylan as carbon source. From each strain 20 µl of a culture medium sample was loaded on gel. *A. niger* transformants, containing copies of the *A. tubingensis axhA* gene, were grown for 20 h (*lanes 1–5*) and 41 h (*lanes 6–10*) are shown in panel **A**: *lanes 1 and 6*: N402; *2 and 7*: NW219::pIM3001-3/11; *3 and 8*: -3/13; *4 and 9*: -15/5; *5 and 10*: -15/13. *A. niger* transformants containing additional copies of the *A. niger axhA* gene, were grown for 24 h (*lanes 1–5*) and 40 h (*lanes 6–10*) are shown in panel **B**: *lanes 1 and 6*: N402; *2 and 7*: NW219::pIM3002-15; *3 and 8*: -28; *4 and 9*: -29; *5 and 10*: -30.

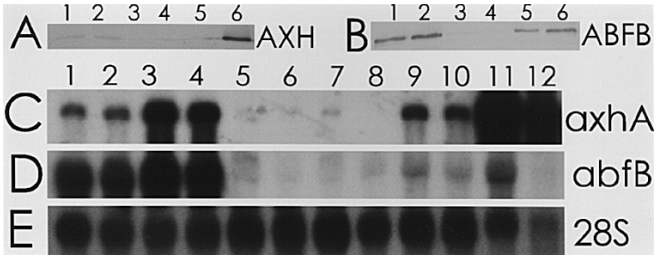

Fig. 3 A–E Functional analysis of the *A. niger axhA* gene. Western analysis of transfer cultures of *A. niger* N402 after 6 h growth. *Lane 1*: 50 mM L-arabinose; *2*: 50 mM L-arabitol; *3*: 50 mM D-fructose; *4*: 50 mM D-glucose; *5*: 50 mM D-xylose; *6*: 1% birchwood xylan; visualized with anti-AXHA **(A)** or anti-ABFB **(B)**. Northern analysis of total RNA of N402 transfer cultures: *lanes 1 and 2*: 50 mM L-arabinose; *3 and 4*: 50 mM L-arabitol; *5 and 6*: 50 mM D-fructose; *7and 8*: 50 mM D-glucose; *9 and 10*: 50 mM D-xylose; *11 and 12*: 1% birchwood xylan after 6 h growth (*lanes 1*, *3*, *5*, *7*, *9* and *11*) and 12 h (*lanes 2*, *4*, *6*, *8*, *10*, *12*). The blots were probed with *axhA* **(C)**, *abfB* **(D)** or 28S rDNA **(E)**

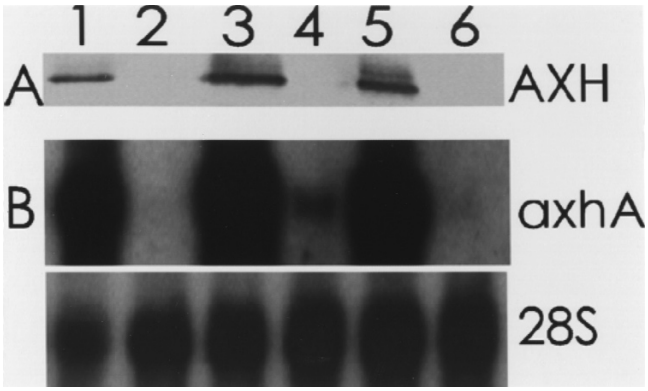

Fig. 4 A,B Functional analysis of *A. niger axhA* in *A. niger* wild-type and strains carrying *creA*^{d}2 or *creA*^{d}4 mutations. Western-blot analysis **(A)** and Northern analysis **(B)** of transfer cultures after 6 h growth of N402 (*lanes 1 and 2*); *creA*^{d}2 (*lanes 3 and 4*) and *creA*^{d}4 (*lanes 5 and 6*) on 1% birchwood xylan (*lanes 1, 3 and 5*) or 1% birchwood xylan/50 mM D-glucose (*lanes 2, 4 and 6*)

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AXHNIG  KCALPSTYSWTSTDALATPKS-GWTALKDFTDVVSNGKHIVYASTTDTQG   75
AXHTUB  KCALPSSYSWSSTDALATPKS-GWTALKDFTDVVSDGKHIVYASTTDEAG   75
XYNC    -CELKAPLRWTSTGPLISPKNPGWISIKDPSIVKYNDTYHVYATYYDTA-  368
         *.* .. .*.**..* .**. **...** . *  .... ***.  *..

AXHNIG  NYGSMGFGAFSDWSDMASASQTATSFS----AVAPTLFYFQPKSIWVLAY  121
AXHTUB  NYGSMTFGAFSEWSNMASASQTATPFN----AVAPTLFYFKPKSIWVLAY  121
XYNC    -YRSM-YTSFTDWNTAQQAPHISMNGSRVGNTVAPQVFYFRPHNKWYLIT  416
         * ** ...*..*.. . *....    .    .*** .***.*.. * *

AXHNIG  QWGSSTFTYRTSQDPTNVNGWSSEQALFTGKISGSSTGAIDQTVIGDDTN  171
AXHTUB  QWGSSTFTYRTSQDPTNVNGWSSEQALFTGKISDSSTNAIDQTVIGDDTN  171
XYNC    QWAGA---YATTDDIRNPN-WSAKQKLLQGE----PNGALDFWVICNDTH  458
         **...   * *..*  * * **..* *. *.    ...*.*  ** .**.

AXHNIG  MYLFFAGDNGKIYRSSMSINDFPGSFGSQYEEILSGATND----LFEAVQ  217
AXHTUB  MYLFFAGDNGKIYRSSMSINDFPGSFGSQYEVILSGARND----LFEAVQ  217
XYNC    CYLYFSRDDGVLYVSKTTLANFP-NFSG-YSIVMEDHRGNGNSYLFEAAN  506
         **.*. *.* .* *. ....** .*.. *. .... ..    ****..

AXHNIG  VYTVDGGEGDSKYLMIVEAIGSTGHRYFRSFTASSLGGEWTAQAA---SE  264
AXHTUB  VYTVDGGEGDTKYLMIVEAIGSTGHRYFRSFTASSLGGEWTAQAA---SE  264
XYNC    VYKLDG---QNRYLLMVEAYISG-----RAFSAPGQRPAWMAHGPLADTE  548
         **.**   ...**..***  *.     *.*.*..  .* *...   .*

AXHNIG  DQPFAGKANSGATWTDDISHGDLVRNNPDQTMTVDPCNLQLLYQGHDPNS  314
AXHTUB  DQPFAGKANSGATWTEDISHGDLVRNNPDQTMTVDPCNLQLLYQGHDPNS  314
XYNC    ANPFAGM---------------MFCFTMASSLKVYTC-----Y-------  571
         ..****.               .   . . ...* .*     *
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Fig. 5 Amino-acid comparison of *A.niger* AXHA (AXHNIG), *A. tubingensis* AXHA (AXHTUB) and *P. fluorescens* XYLC (XYLC). Amino-acid sequences were aligned using the program CLUSTAL (Intelligenetics). Identical amino acids are shown by '*' and similar amino acids are shown by '.'

results. Transcription of *abfB* was strongly induced by L-arabitol and L-arabinose and only weakly by xylan (Fig. 3, panels b and d).

To study the effect of carbon catabolite repression on AXHA expression, two *A. niger* strains carrying *creA*^{d} mutations (van de Vondevoort, unpublished results) and the wild-type were pre grown on 100 mM of D-fructose and transferred to minimal medium containing either 1% (w/v) birchwood xylan or 1% (w/v) birchwood xylan in combination with 50 mM of D-glucose and cultured for another 6 h. Both Northern and Western analysis gave similar results: AXHA expression was de repressed in the *creA*^{d}2 and *creA*^{d}4 mutant strains compared to wild-type when grown on xylan, whereas AXHA expression levels were very low both in wild-type and in the *creA* mutant strains when grown on 1% xylan in combination with 50 mM of D-glucose (Fig. 4).

Discussion

The nucleotide sequences of the genes encoding AXHA in *A. niger* and *A. tubingensis* have been determined and comparison with the EMBL and GenBank databases did not result in the identification of significant primary sequence similarities. However, comparison of the amino acid sequences with the SWISSPROT database revealed a low, but significant, homology with XYLC from *Pseudomonas fluorescens* subsp. *cellulosa*, which is an α-L-arabinofuranosidase acting only on oat spelt xylan (Kellett et al. 1990). XYLC consists of a cellulose-binding domain and a catalytic domain. Both AXHA mature proteins show 32% identity with this catalytic domain of XYLC (Fig. 5).

The major *tsp*, 64 bp upstream of the putative start codon, is preceded in both genes by a 13 bp CT-rich element TTATTCGTTCTCPy. Pyrimidine-rich tracts or CT boxes are frequently observed in promoters of filamentous fungal genes and have been postulated to function in the correct initiation of transcription (Punt et al. 1990). The 5′ non-coding regions of the *A. tubingensis* and *A. niger axhA* genes contain one sequence motif, 167 bp upstream of the major *tsp* (numbering refers to the *A. niger* sequence), perfectly matching the CREA consensus sequence (G/C C/T G G G G). This element is involved in D-glucose-mediated carbon catabolite repression by the DNA-binding protein CREA (Kulmburg et al. 1993).

Two pairs of inverted repeats, conserved in both *axhA* sequences, were found 170/142 bp and 134/102 bp upstream of the major *tsp*, respectively. Whether these repeats are functional in vivo remains to be investigated, since none of these sequence elements could be found in the promoters of other xylanolytic genes in *Aspergillus*. Downstream from the proposed stop codon, the mRNA of *A. niger axhA* contains a non-coding region which can vary in size, viz. 172 and 183 bp. This suggests the presence of (at least) two polyadenylation sites, which are frequently observed for other fungal genes (Gurr et al. 1987). Although a few AT-rich sequences were found in the 3′ non-coding region of both genes, a consensus polyadenylation signal was not present (Proudfoot 1991).

Comparison of the two deduced amino acid sequences revealed differences at 21 positions. Eleven of these amino acid substitutions arise from single point mutations. In addition to the mutations leading to amino acid substitutions, 76 silent mutations were found. The close relationship between *A. niger* and *A. tubingensis* is illustrated by comparing their respective *axhA*, endo-xylanase B (*xynB*), polygalacturonase II

Table 1 Homology between the *A. niger* and *A. tubingensis axhA*, *xynB*, *pgaII* and *pme* genes

Item	*axhA*[a)]	*xynB*[b)]	*pgaII*[c)]	*pme*[d)]
Amino-acid identity	94%	92%	94%	98%
Nucleotide identity				
Coding sequence	89%	91%	90%	96%
Intron(s)	–	69%	79%	75%
5′ non-coding	80%	78%	81%	95%
3′ non-coding	70%	78%	82%	74%

[a)] This study
[b)] Kinoshita et al. (1995); Ito, K, unpublished results (Gen Bank accession No. D38070)
[c)] Bussink et al. (1991)
[d)] Kusters-van Someren and Visser, unpublished data

(*pga*II) and pectin methyl esterase (*pme*) genes. As shown in Table 1, the nucleotide identity between the *axhA* genes is comparable to those of the other three systems (Visser et al. 1996). In addition to a comparison of the DNA sequence data, the black *Aspergilli* can also be classified on the basis of RFLP patterns and biochemical features, such as mobility on SDS-PAGE or isoelectric focusing. The *axhA* gene can be used as a probe in RFLP analysis of chromosomal DNA, since the restriction maps of the *A. niger* and *A. tubingensis axhA* genes and their flanking sequences show significant differences (Fig. 1).

Although both AXHA and ABFB are active on arabinoxylan, the expression of these two L-arabinose releasing activities is regulated differently as shown by the transfer experiments. The results also demonstrate that the expression is transcriptionally regulated. Whereas ABFB expression was strongly induced by L-arabitol and L-arabinose, AXHA expression was strongly induced by xylan and much less by L-arabitol, L-arabinose and D-xylose, as shown in Fig. 3. Expression of *axhA* is possibly regulated in a similar manner to that proposed for the expression of *xlnA* in *A. tubingensis* (de Graaff et al. 1994). *AxhA* transcription is most likely under direct control of a route-specific transcriptional activator. The Northern analysis shown in Fig. 4 demonstrated that carbon catabolite repression of *axhA* transcription is controlled at two levels, i.e. directly by repression of *axhA* transcription, and indirectly by repression of the expression of a transcriptional activator. The direct involvement of *creA* is demonstrated by increased *axhA* transcription in the *creA*^{d} mutants under inducing conditions. However, when grown on xylan and D-glucose, *axhA* transcription is still repressed both in wild-type and *creA*^{d} mutant strains as a result of non-*creA* mediated carbon catabolite repression effects on the expression of the transcriptional activator. This type of mechanism would be similar to the mechanism for the regulation of gene expression of *xlnA* in *A. tubingensis* (de Graaff et al. 1994).

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