A molecular analysis of (hemi-)cellulose degradation by Aspergilli

Marco Gielkens

Promotor:	dr. ir. A. J. J. van Ooyen Hoogleraar genetische technieken in de levensmiddelentechnologie
Co-promotor:	dr. ir. L. H. de Graaff Universitair docent, sectie moleculaire genetica van industriële micro-organismen

Markus Matheus Catharina Gielkens

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Contents

Chapter 1

General introduction.

Introduction

The cell wall provides rigidness to the living plant and protects it against physical damage and microbial attack. The cell wall is composed of approximately 90% of polysaccharides and 10% proteins. The three main polysaccharides that have been identified are celluloses, hemicelluloses like arabinoxylans, and pectic polysaccharides [39]. Basically, the primary cell wall is a network of cellulose microfibrils. These microfibrils consist of several dozens linear chains of β-1,4-linked D-glucose molecules which are condensed to form long crystalline structures that wrap around each cell. The principal polymers that interlock the microfibrils are (glucurono)arabinoxylans, linear chains of β-1-4-linked D-xylose molecules, which can be substituted with single L-arabinose units and less frequently, single glucuronic acid units [10]. When growing in their natural habitat, saprophytes as well as phytopathogens are able to secrete a broad range of enzymes that can degrade these complex polymers. This is necessary since the cell cannot directly take up these large polymers. Filamentous fungi, such as *Aspergillus niger* and *Trichoderma reesei*, are capable of secreting cellulolytic, xylanolytic and pectinolytic enzymes. Despite the fact that many glycosylhydrolases and their encoding genes have been isolated and characterised, little is known how microorganisms regulate their polysaccharide-degrading enzyme systems to degrade plant cell wall materials to metabolisable carbon sources.

Regulation of gene expression

In general, the expression of polysaccharide degrading enzymes is controlled by induction *via* a specific transcriptional activator and by carbon catabolite repression. Carbon catabolite repression is a global regulatory mechanism, in which the presence of rapidly metabolisable carbon sources represses the expression of genes involved in the utilisation of less-favoured carbon sources. This enables the microorganism to use the energetically most favourable carbon source and not waste energy on the synthesis of other catabolic systems.

The general concept for regulation of expression and secretion of polysaccharide degrading enzymes is that low constitutive levels of particular enzymes are secreted and released in the medium or are bound to the conidial surface. These enzymes produce 'signal molecules' if certain polymers are present. The 'signal molecules' are taken up by the cell, where they may be converted or act directly as an inducer for synthesis of a subset of enzymes required to degrade the polymer. This model is largely based on the induction of the cellulolytic system of *T. reesei* [9,47]. Signal molecules that have been shown to function in Aspergilli are monomeric sugars, or products thereof formed intracellularly, like D-xylose in the case of the *Aspergillus tubingensis xlnA* gene encoding an endoxylanase [22] and similarly L-arabinose and L-arabitol act as inducers for the arabinan degrading system of *A. niger* [64]. A second class of signal molecules are homo- and heterosaccharides, which can be formed as degradation products or by transglycosylation reactions. In *Aspergillus terreus* the heterodisaccharide D-glucose-β-1,2-D-xylose induces both cellulolytic and xylanolytic enzymes, whereas homodisaccharides induce selectively cellulases (e.g. sophorose = D-glucose-β-1,2-D-glucose) or xylanases (*e.g.* D-xylose-β-1,2-D-xylose)[25]. The presence of an inducer activates an induction pathway, which results in the synthesis of the enzyme system. In principle, this pathway consists of an uptake system for the inducer which may or may not be constitutive, and of a transcriptional activator, since the expression of the polysaccharide degrading enzymes is regulated at the level of transcription. The transcriptional activator drives directly or indirectly the transcription of the structural genes encoding the polysaccharidases.

Xylan degradation

Xylan is next to cellulose the most abundant polysaccharide present in the plant cell wall. It is a heteropolymer consisting of a backbone of β-1,4-linked D-xylose residues which can be modified by various substituents: 1,2-linked α-D-glucuronic acid or 4-O-methyl-α-D-glucuronic acid residues can be present, as well as 1,2- and 1,3-linked α-L-arabinose residues. In some cases these L-arabinose residues are esterified with ferulic and *p*-coumaric acid, enabling cross-linking of the xylan to the lignin-matrix. Depending

on the source of the plant material, the D-xylose residues in the backbone can be modified by acetylation at the C-2 or C-3 position [72].

Complete degradation of this heteropolymer requires the synergistic action of a spectrum of enzymes (Fig. 1). Enzymes involved in xylan degradation are endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), acetylxylan esterase (EC 3.1.1.72), L-arabinose releasing enzymes such as α-L-arabinofuranosidase (EC 3.2.1.55) and arabinoxylan arabinofuranohydrolase, α-glucuronidase (EC 3.2.1.139), feruloyl esterase, and *p*-coumaroyl esterase [7]. Recently, enzymes have been found that have a high specificity towards arabinoxylan or arabinoxylan derived oligosaccharides and which are active against L-arabinofuranosyl groups linked to double-substituted D-xylopyranosyl residues [34].

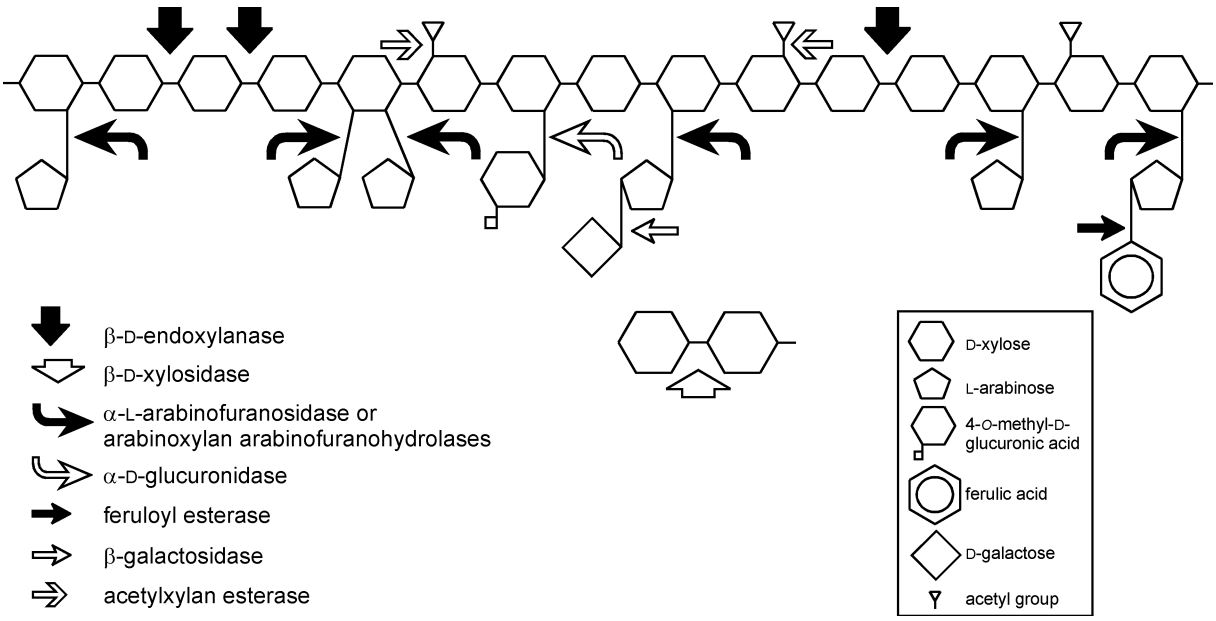

Fig. 1. Arabinoxylan structure and enzymes involved in arabinoxylan degradation (adapted from Biely)[7].

Endoxylanases attack xylan at the D-xylose backbone thus rapidly lowering the degree of polymerisation. Most xylanases are strongly inhibited in their activity by side groups attached to the D-xylose backbone, *e.g.* acetyl groups, L-arabinose or D-glucuronic acid residues. Accessory enzymes, like AxeA, AxhA or AguA, which remove these side groups, have been shown to have strong synergistic effect with endoxylanases, thus facilitating the complete degradation of the heteropolymer [31]. Synergy is the

enhancement of activity over the added activities of the individual enzymes. The xylo-oligosaccharides are further hydrolysed by β-xylosidase.

Several genes encoding arabinoxylan degrading enzymes have been cloned from *A. niger*, *viz.* two endoxylanase genes *xlnB* [28] and *xlnC* [20], a β-xylosidase gene *xlnD* [42], an α-L-arabinofuranosidase gene *abfB* [19], an arabinoxylan arabinofuranohydrolase gene *axhA* [21], an acetylxylan esterase gene *axeA* [23], an α-glucuronidase gene *aguA* [69] and a feruloyl esterase gene *faeA* [68]. *A. tubingensis*, which is closely related to *A. niger* has a third endoxylanase encoding gene *xlnA*, which is not present in *A. niger* [22].

Induction of the xylanolytic system in *A. niger*

The induction pathway of an extracellular enzyme system consists in principle of an uptake system for the inducer and a transcriptional activator that drives the transcription of structural genes. The polymer is too large to be taken up directly by the cell. It is therefore assumed that initial degradation products of the polysaccharide induce the expression of the enzymes, which are involved in its further hydrolysis.

In the case of xylan, these degradation products are D-xylose and probably also small xylo-oligosaccharides. Not much is known about the uptake system for the inducer in *A. niger*. Both D-xylose and xylobiose are thought to be taken up by the fungus and act as an inducer. More is known about the formation of the inducing compounds. The *A. niger* β-xylosidase, encoded by *xlnD*, could have an important role in xylanolytic inducer formation. The enzyme has been shown to be active towards xylan and xylo-oligosaccharides resulting in the formation of D-xylose, which is shown to be a good inducer of xylanolytic enzymes in *A. niger* [22,42,44,68,69,70]. Moreover, transglycosylation products of D-xylose by β-xylosidase have been implicated to play a role in induction [48]. Northern blot analysis revealed no significant differences in the expression of xylanolytic genes between wild-type and a *xlnD* disruption mutant when these strains were grown on D-xylose or xylan. Therefore, β-xylosidase is not necessary for inducer formation and induction during growth on xylan and D-xylose. These data also show that induction does not result from transglycosylation reactions catalysed by β-xylosidase [42].

The second component of the induction pathway is the specific transcriptional activator. The *A. tubingensis xlnA* gene proved to be an essential tool for the cloning of the gene encoding the xylanolytic transcriptional activator protein XlnR in *A. niger*. After conducting promoter deletion analysis experiments, a region was identified containing an upstream activating element (UAS) involved in xylan-specific induction of *xlnA* in *A. tubingensis*. This fragment was cloned in front of a reporter gene and it was shown that both xylan and low-molecular-weight carbon sources, *e.g.* D-xylose, were able to induce expression of xylanase genes and the reporter gene. It was found that both induction and carbon catabolite repression are mediated *via* this fragment. Thus, it was hypothesised that carbon catabolite repression acts both directly on the *xlnA* gene and indirectly by repressing transcription of the route-specific transcriptional activator (Fig. 2)[22].

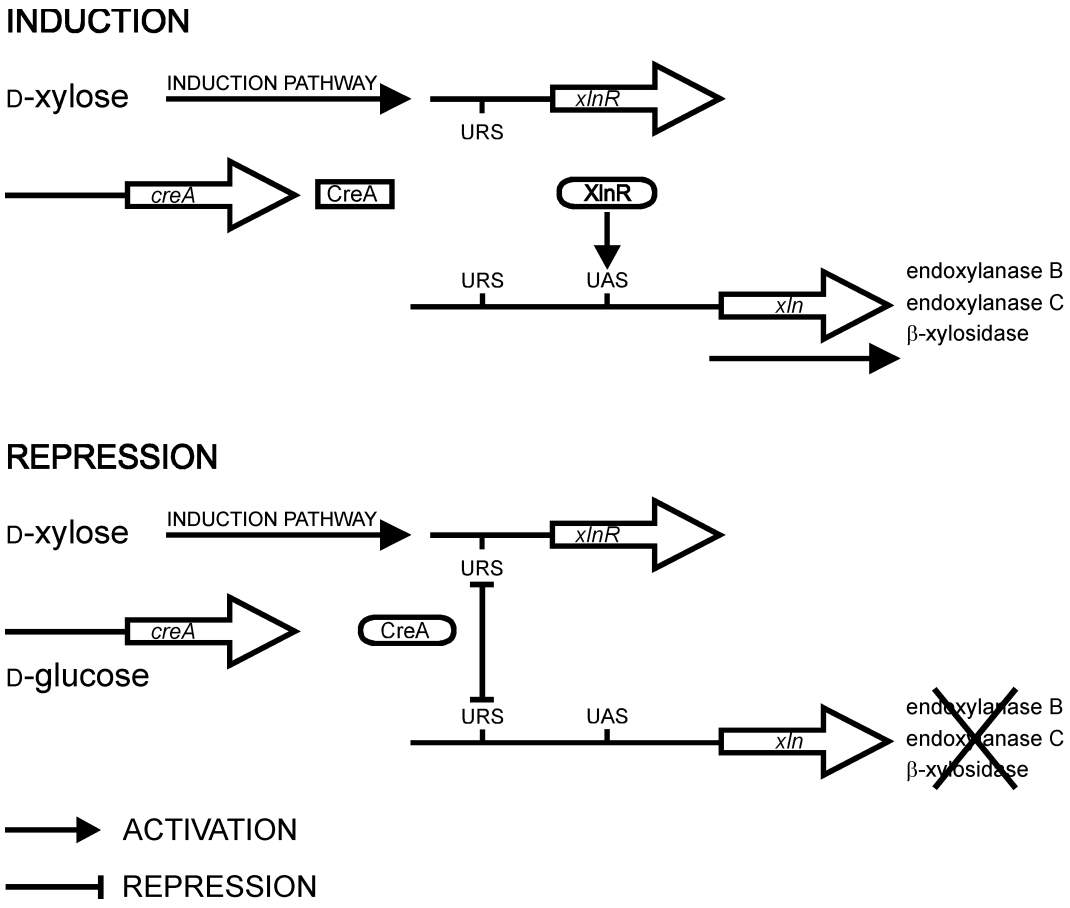

Fig. 2. Schematic model for the regulation of the genes involved in degradation of xylan of *A. niger*. The model assumes the requirement of a transcriptional regulator XlnR encoded by the *xlnR* gene to activate the transcription of the structural genes (upper panel). Under conditions of carbon repression both the structural gene as well as the *xlnR* gene are repressed by CreA (lower panel)[43].

The next step was to clone a 158 bp fragment containing the UAS in front of the bi-directional marker gene *pyrA, i.e.* for a positive PYR^+ (uridine prototrophic) or a negative PYR^- (fluoro-orotic acid resistant) phenotype, under conditions with a normally opposite phenotype. By controlling expression using this regulated promoter fragment, it was possible to select for xylanolytic regulatory mutants, designated as NXA (non-xylanase producers). Ten of these mutants showed poor growth on xylan as sole carbon source. Growth experiments in liquid cultures using D-xylose or xylan as carbon source showed that the isolated mutants had strongly decreased endoxylanase and β-xylosidase activities compared to the wild-type strain, while the expression of α-L-arabinofuranosidase activity was not affected. This indicated a mutation in a *trans*-acting factor involved in the expression of endoxylanase and β-xylosidase [44]. The uridine auxotrophy of the NXA mutants on D-xylose was used to clone the complementing gene. Sequence analysis of the isolated *xlnR* gene revealed the presence of a zinc binuclear DNA-binding domain in the protein encoded. DNaseI footprint analysis and comparison of xylanolytic promoter sequences led to the identification of the sequence 5'-GGCTAA-3' as the binding site of XlnR. Mutational analysis demonstrated the second G to be essential for functionality of the UAS [44].

The availability of both the *xlnR* gene and the *A. niger* XlnR loss-of-function mutants gave the opportunity to study the spectrum of genes being controlled by XlnR at the transcriptional level. Northern blot analysis was conducted to study the transcription of genes which are transcriptionally regulated by XlnR in an *A. niger* wild-type strain, an *xlnR* loss-of-function mutation and a strain containing multiple copies of the *xlnR* gene. From the results it was concluded that XlnR not only activates transcription of the genes encoding the main xylanolytic enzymes, such as *xlnB*, *xlnC* and *xlnD*, but also genes encoding accessory enzymes involved in the xylan degradation (Chapter 5). The elevated expression of *aglB* and *lacA*, encoding α-galactosidase B and β-galactosidase A, respectively, observed on xylan and D-xylose could also be assigned to regulation via XlnR [67]. Moreover, some genes involved in the degradation of cellulose are also transcriptionally activated by XlnR indicating that the transcriptional regulation by XlnR is not to restricted the genes encoding xylanolytic enzymes and includes regulation of two endoglucanase encoding genes and two cellobiohydrolase encoding genes. (Chapters 5 and 6). These results emphasise the key role of XlnR in the induction of hemicellulose and cellulose degrading enzymes.

Cellulose degradation

Cellulose, the major component of plant cell wall, is a linear polysaccharide composed of 30-15,000 β-D-glucopyranosyl units linked by β-1,4-glucosidic bonds. Cellulose is arranged in fibres that have a fully extended flat conformation and are tightly packed into microfibrils by hydrogen bonds to form an insoluble fibrous material. These insoluble fibres are also known as microcrystalline cellulose and they can be interrupted by short amorphous regions [30]. The complete enzymatic hydrolysis of cellulose to D-glucose requires the action of at least three different types of cellulases: endo-β-1,4-D-glucanase (EC 3.2.1.4), exo-β-1,4-D-glucanase or exo-β-1,4-D-cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). The cellobiohydrolases (Cbh) are subdivided into two classes. CbhI enzymes attack cellulose at the reducing ends of cellulose chains, whereas CbhII enzymes attack cellulose at the non-reducing end [60]. For both enzymes this results in the release of cellobiose. Recently, a novel exoglucanase I (ExoI) was isolated from the cellulolytic fungus *Humicola grisea*. Although this protein has some structural similarities to CbhI proteins, its activity results in the release of D-glucose instead of cellobiose [58].

Because of its structural rigidity, crystalline cellulose is resistant to the action of individual cellulases. Effective conversion of cellulose to monosaccharides is therefore only possible by the synergistic action of these enzymes (Fig. 3)[73]. As cellulose is a natural polymer, its crystallinity is rarely perfect. Amorphous regions occur in particular near the crystal surfaces and are prone to enzymatic attack. Endoglucanases are thought to attack in the middle of the more disordered regions of cellulose and the cellobiohydrolases attack the crystalline areas at the opposite chain ends. These two types of enzyme activities are most effective in the so-called endo-exo synergism. A simplified model of endo-exo synergism is that the endoglucanases hydrolyse the cellulose internally, thereby producing more free ends to which the cellobiohydrolases can bind and start to hydrolyse. The synergism observed is thus due to the increase in substrate for the cellobiohydrolases. Also, cellobiohydrolases act progressively along the cellulose chain thereby loosening the cellulose chain from the microfibril and exposing new sites of attack for endoglucanases [60]. The smaller cello-oligosaccharides are further hydrolysed by cellobiohydrolases and β-glucosidases.

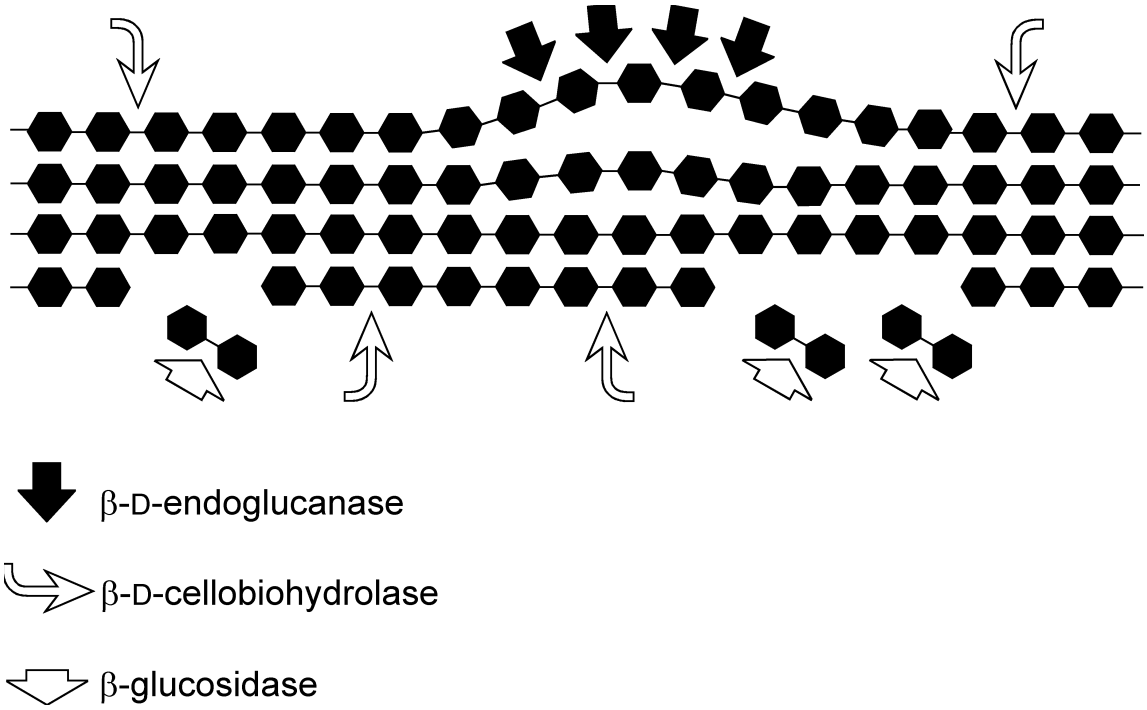

Fig. 3. Cellulose structure and enzymes involved in cellulose degradation.

The best studied fungal cellulolytic system is that of *T. reesei*. Eight genes encoding different enzymes have been cloned. Two major cellobiohydrolase genes, *cbh1* [54,62] and *cbh2* [11,61], two major endoglucanase genes *egl1* [1,45] and *egl2* (originally called *egl3*)[52], and a β-glucosidase encoding gene *bgl1* [6]. Three additional endoglucanase genes encoding minor activities were found later; *egl3* [67], *egl4* [53] and *egl5* [50]. With the exception of EglIII and BglI, all the *T. reesei* cellulases have a modular structure. This modular structure consists of a catalytic domain and a cellulose binding domain (CBD) separated by a linker peptide rich in serine, threonine and proline residues. The CBD is a well-conserved domain and is found at either the N-terminus or C-terminus. In *T. reesei* two hemicellulases, an endo-mannanase [55] and an acetyl-xylan esterase [38], have been found which also have a modular overall architecture with a C-terminal CBD. This feature is also common for all the cellulases and hemicellulases cloned from the prokaryote *Pseudomonas fluorescence* subsp. *cellulosa* (see [24] for review). The exact role for CBD and linker sequences has yet to be established. One proposed function of the CBD is the release of cellulose chains from the cellulose crystal prior to hydrolysis by the catalytic domain thereby increasing the activity of cellulases against the more resistant forms of insoluble cellulose [29]. Removal of the CBD has

little effect on the activity of cellulases towards soluble substrates, whereas the activity towards insoluble substrates is clearly decreased [36]. The CBD therefore enhances the catalytic activity by promoting close contact between the catalytic domain and its target substrate, *i.e.* by lowering the effective K_m of the enzyme for natural substrates. Cellulases with CBDs are required in the early stages of cellulose degradation when most of the substrate is still insoluble. At later stages, when most of the substrate has been solubilised, enzymes without CBDs might be preferred. In *T. reesei*, it has been shown that in later stages of cellulose degradation the CBD is proteolytically removed from EglII [46] and it is thought that this same mechanism also applies for other cellulases including cellobiohydrolases. The proteolytic removal of the linker peptide and the CBD may serve as an *in vivo* mechanism to alter the properties of cellulases during hydrolysis, when complex insoluble substrates are gradually shortened to soluble and more accessible substrates [46]. Recently several genes have been cloned which lack a CBD and consist of only a catalytic domain. One is the above-mentioned exoglucanase (ExoI) from *H. grisea* [58]. The gene product of *Phanerochaete chrysosporium cbh1-1* also lacks a hinge region and a CBD. The white rot fungus *P. chrysosporium* has multiple genes exhibiting significant homology to the *T. reesei cbh1* gene. Among these genes *cbh1-1* is the only gene lacking a CBD [13]. These organisms express, in contrast to most cellulolytic fungi, two or more genes encoding similar activities, however, some with and some without a linker peptide and CBD. These organisms utilise a different strategy to achieve the same goal, since they are able to synthesise cellobiohydrolases with and without a CBD.

Induction of the cellulolytic system

Crystalline cellulose can not be taken up by fungi directly and therefore needs to be degraded to mono- and/or oligosaccharides by cellulases secreted by the fungus. Despite the progress that has been made in the characterisation of *T. reesei* cellulase systems, it is still poorly understood how the biosynthesis of cellulases is triggered by the extracellular and insoluble cellulose. On the one hand, it was long hypothesised that the fungus contains low, constitutive cellulase levels, which may attack cellulose when available and thus lead to the formation of the inducer activity of cellulase biosynthesis. It has been

shown that this is indeed true for the *cbh1* and *egl1* genes in *T. reesei*. Transcripts of these genes are present in low levels in the non-induced conditions and are induced at least 1100-fold in the presence of cellulose [9]. On the other hand there are reports that conidial bound CBHII is responsible for the initial attack on cellulose and the subsequent release of inducer [40]. According to both mechanisms, the major end product` formed by the initial attack is cellobiose. This disaccharide is the first soluble compound released from cellulose, and its appearance in the cell should specifically indicate the presence of extracellular cellulose. Cellobiose could therefore be the natural inducer of further cellulase biosynthesis. And indeed, there are several microorganisms known in which the biosynthesis of cellulases can be induced by cellobiose [8,25,26,57]. However, the transglycosylation product of cellobiose, sophorose, was found to be a much stronger inducer of cellulases in both *T. reesei* and *A. terreus* [25,26]. Both BglA and EglI are known to form sophorose by transglycosylation [12,65]. In *T. reesei*, cellobiose can induce cellulase biosynthesis in comparable amounts as cellulose when it is slowly fed or when cellobiose hydrolysis by β-glucosidase is inhibited. This suggests that the cellobiose concentration is critical in determining whether cellobiose can act as an inducer or not, and it may do so only if it is not hydrolysed by β-glucosidase. Both sophorose and cellobiose can be transported rapidly into the cells by a β-diglucoside permease. The affinity of this permease towards cellobiose is much higher than that of β-glucosidase, whereas the maximal activity is much lower. It is therefore assumed that the induction of cellulases by slow feeding of cellobiose is due to a preferred uptake by the permease if the concentration of cellobiose is low [32]. The reason why sophorose acts as a stronger inducer than cellobiose is not well understood. One explanation is that sophorose is less rapidly metabolised by the fungus and can therefore exert induction more effectively.

Limited data are available regarding a putative transcriptional activator which regulates the transcription of genes encoding cellulolytic enzymes. Studies using *T. reesei* mutants defective in cellulose induction suggested a mechanism of co-ordinately regulating the expression of cellulase genes. It was proposed that these mutants are most probably defective in (a) transcription factor(s) shared by these cellulase-encoding genes, which is required for induction and/or the uptake of the soluble inducer formed on cellulose [63]. An 11 bp element was found in the promoter of the *T. reesei cbh2* gene which was designated as *cbh2*-activating element (CAE). This element is responsible for

the formation of protein complexes from cellulase-forming (induced) and non-induced mycelia and is essential for induction of gene expression on sophorose and cellulose *in vivo*. CAE consists of a 5'-CCAAT-3' and a 5'-GTAATA-3' binding motif which probably interacts with a specific transcriptional activator. The 3' region bears some resemblance to the *A. niger* XlnR binding site. However competitive binding studies using oligonucleotides derived from the *A. niger xlnD* promoter demonstrated that the protein binding to the 3' fragment is not the XlnR homologue in *T. reesei* [74]. To date there are two putative transcriptional activators known (AceI and AceII) which were isolated with the yeast one-hybrid cloning system using the promoter of the *T. reesei cbh1* gene. AceII contains a zinc binuclear DNA-binding domain and was reported to bind to the DNA sequence 5'-GGCTAATAA-3'resembling the *A. niger* XlnR binding site, however its primary structure differs significantly from XlnR [51]. At this moment it is not clear if one or both of these regulators activate the transcription of genes encoding other cellulolytic or non-cellulolytic enzymes.

Carbon catabolite repression

The degradation of large and heterogeneous polysaccharides into monomeric sugars is a complex process in which a wide variety of (hemi-)cellulolytic enzymes is involved. It is therefore important for the fungus to synthesise these enzymes only when they are necessary. In the presence of readily metabolisable sugars, such as D-glucose, the synthesis of polysaccharidases is repressed due to carbon catabolite repression. This is a global regulatory mechanism in fungi, such as *Aspergilli* and *T. reesei*, by which, in the presence of rapidly metabolisable carbon sources, the expression of genes involved in the utilisation of less-favoured carbon sources is repressed. From the viewpoint of cellular physiology this is beneficial for two reasons. Firstly, the energetically most favourable carbon source is used and secondly, no energy is wasted on the synthesis of other catabolic systems [49]. It is now clear that in *Aspergilli* the repressor protein CreA plays a major role in carbon repression. CreA inhibits transcription of many target genes by binding to specific sequences in the promoters of these genes.

A. nidulans strains carrying *creA* mutant alleles were isolated using *areA* loss-of-function mutants with compounds which can act both as carbon and nitrogen source [3].

AreA, a wide-domain regulator protein for nitrogen metabolism, is required for expression of genes related to utilisation of other nitrogen sources than ammonia or glutamine. Some of these alternative nitrogen sources, *e.g.* proline or acetamide, are controlled by nitrogen and carbon repression as they can serve both as nitrogen and carbon source. Relief of either nitrogen or carbon repression permits utilisation of these compounds, but growth on a combination of a repressing nitrogen source and a repressing carbon source completely blocks proline and acetamide catabolism. Therefore *areA* null mutants are unable to utilise proline as a nitrogen source in the presence of a repression carbon source, such as D-glucose. Arst and Cove [3] used this phenotype to isolate mutations that interfere with carbon repression. These mutations are designated as *creA*^{d} mutations. Another strategy makes use of *A. nidulans pdhA* mutants, lacking a functional pyruvate dehydrogenase complex. A number of *creA* mutants were isolated as pseudo-revertants of *pdhA* on media containing D-glucose and ethanol [5]. The *creA* gene thus identified was thought to be a negatively acting wide domain regulator [2,4]. The *A. nidulans creA* gene has been cloned and characterised by Dowzer and Kelly [15,16]. DNase I footprinting experiments have led to the definition of a binding site for CreA, 5'-SYGGRG-3' [33]. However, not all possible sequences included in this consensus are always functional, *i.e.* the binding affinity for certain variants is context-dependent on the sequences outside the hexanucleotide [14]. The *A. niger creA* gene [17], the *T. reesei* homologue *cre1* [27,56], and the *creA* gene from the cellulolytic fungus *H. grisea* have also been cloned [59].

In the case of the ethanol regulon of *A. nidulans* carbon catabolite repression operates at two levels. It represses both *alcR*, encoding the transcription activator for *alc* genes and the structural genes *alcA*, encoding alcohol dehydrogenase I and *aldA*, encoding aldehyde dehydrogenase. Thus, the ethanol catabolism is carbon repressed by a double-lock mechanism as proposed by Felenbok. [18]. This model for regulation of the *alc* system might be valid in general for catabolic systems. Carbon repression mediated by CreA has also been observed in genes encoding xylanolytic and cellulolytic enzymes [22,37,41,46,57,58,59].

Recently it has been shown that in *A. niger* CreA modulates the XlnR-induced transcription of genes encoding xylanolytic enzymes when the fungus is grown on D-xylose. The transcription of the *xlnB*, *xlnD*, *aguA* and *faeA* genes on D-xylose was studied in a wild-type strain and in a *creA*^{d} mutant. A decrease was observed in transcription

levels of all four genes with increasing D-xylose concentrations, whereas the transcription levels were unaffected in the *creA*^{d} mutant strain. The results indicated that the transcription levels of these xylanolytic genes were partially repressed at D-xylose concentrations higher than 1 mM [70]. These results demonstrate that studies on the effect of D-xylose on the xylanolytic system in fungi, and in particular *A. niger*, should not only focus its role as an inducer, but also as a factor triggering carbon repression. Thus, both a specific regulator and the CreA repressor protein regulate transcription of these genes. Presence and concentration of the carbon source determine the balance between induction and repression controlled by these regulatory proteins. This is also illustrated by the influence of D-glucose concentrations on the regulation of cellulase biosynthesis, as the end-product of cellulose hydrolysis, D-glucose, inhibits further synthesis of cellulases. In *T. reesei*, it has been shown that D-glucose interferes with cellulase biosynthesis by blocking the uptake of diglucosides that can act as an inducer [32] and by repression of *de novo* biosynthesis of cellulases *via* the transcriptional repressor protein Cre1. However the underlying mechanisms need still to be unravelled.

Aim and outline of this thesis

Glycosylhydrolases as cellulases and xylanases are of great importance for the ecological recycling of biomass. In addition, a whole range of commercial enzyme preparations containing fungal polysaccharidases is used in industrial applications. For example, xylanases and cellulases are used in the food and feed industry, in pulp and paper applications, such as improvement of bleachability of pulp and lowering of the pulp viscosity [66], whereas cellulases are also used in the textile industry in biostoning applications [35]. The aim of this thesis is to clone and study regulation of genes encoding novel activities capable of degrading (hemi-)cellulose. Many of these novel activities are minor activities, which are difficult to detect and identify using standard conditions for growth. Several strategies will therefore be exploited to find these novel activities in Aspergilli. Firstly, the conditions of induction need to be optimal. This involves both the carbon source as well as the duration of growth (Chapter 4). Secondly, accumulation of the inducer often results in higher levels of expression (Chapter 3). Also the use of derepressed *creA*^{d} strains can result in elevated levels of expression as is

shown in Chapters 2 and 3. Chapter 3 also shows that gene disruption of the major α-L-arabinofuranosidase encoding gene can reveal minor activities which are otherwise difficult to identify. Finally, increasing the gene dosage of a specific transcriptional activator can increase the transcription levels of genes controlled by that activator (Chapters 5 and 6). Despite the fact that many glycosylhydrolases and their encoding genes have been isolated from a wide variety of microorganisms, little is known at the molecular level about the factors that are involved in the expression of these genes. The research described in this thesis gives a better understanding of the mechanisms underlying the regulation of expression of genes encoding cellulose- and hemicellulose-degrading enzymes produced by *Aspergillus*.

Chapter 2 describes the isolation of *A. niger creA*^{d} mutants relieved of carbon repression and the effects of the mutations `on the expression of arabinanases and L-arabinose catabolic enzymes. This system was selected to illustrate that the expression of genes involved in the utilisation of less-favoured carbon sources could be enhanced in derepressed *creA*^{d} mutant strains. Chapters 3 and 4 focus on genes encoding enzymes that are able to release L-arabinose from arabinoxylan. Chapter 3 describes the cloning and characterisation of the *A. nidulans abfB* gene, as well as the analysis of expression of the L-arabinofuranosidase encoding gene in an *A. nidulans* wild-type strain and several mutant strains. Super-induction of the *abfB* gene can be accomplished by combining a mutation leading to the intracellular accumulation of an inducer with a *creA*^{d} mutation, as is shown by expression analyses. This chapter also describes the identification of minor L-arabinose releasing activities when the major α-L-arabinofuranosidase activity was disrupted in a derepressed *creA*^{d} genetic background. The cloning, characterisation and analysis of expression of the *axhA* genes from the closely related fungi *A. niger* and *A. tubingensis* is described in Chapter 4. These genes encode an arabinoxylan-arabinofuranohydrolase A enzyme which specifically releases L-arabinose substituents from arabinoxylan. This chapter also describes the transcriptional analysis of the *axhA* and *abfB* genes in *A. niger*. It demonstrates that the regulation of transcription of the two genes differs significantly, although both genes encode L-arabinose releasing activities. The transcription of genes encoding enzymes involved in xylan degradation and two endoglucanases involved in the degradation of cellulose in *A. niger* is studied in Chapter 5. In particular, the role of the transcriptional activator XlnR in the regulation of transcription of these genes was investigated. This analysis is extended in Chapter 6,

which describes the cloning and characterisation of two cellobiohydrolase encoding genes in *A. niger*, *cbhA* and *cbhB*, which are also involved in the degradation of cellulose. The results described in Chapters 5 and 6 illustrate that the range of genes transcriptionally regulated by a specific activator is not necessarily restricted to genes encoding pathway-specific enzymes but that it also includes genes encoding non-pathway specific activities. Furthermore, the data described in these chapters give evidence that increasing the gene dosage of a specific transcriptional activator could elevate the expression of a broad range of genes controlled by that activator. Finally, the results presented in this thesis are summarised and discussed.

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Chapter 2

Isolation of *Aspergillus niger creA* mutants: Effects on expression of arabinanases and L-arabinose catabolic enzymes.

G.J.G. Ruijter, S.I. Vanhanen, M.M.C. Gielkens,

P.J.I. van de Vondervoort, J. Visser

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ABSTRACT

Aspergillus niger **mutants relieved of carbon repression were isolated from an *areA* parental strain by selection of strains that exhibited improved growth on a combination of 4-aminobutanoic acid (GABA) and D-glucose. In addition to derepression of the utilisation of GABA as a nitrogen source in the presence of D-glucose, three of the four mutants also showed derepression of L-alanine and L-proline utilisation. Transformation of the mutants with the *A. niger creA* gene, encoding the repressor protein CreA, re-established the *areA* phenotype on GABA/D-glucose, identifying the mutations as *creA*^{d}. The *creA* gene mapped on chromosome IV by linkage analysis and CHEF hybridisation. The *creA* mutants obtained were used to study the involvement of CreA in repression by D-glucose of arabinanases and L-arabinose catabolism in *A. niger*. In wild-type *A. niger* α-L-arabinofuranosidase A, α-L-arabinofuranosidase B, endo-arabinanase, L-arabinose reductase and L-arabitol dehydrogenase were induced on L-arabinose, but addition of D-glucose prevented this induction. Repression was relieved to varying degrees in the *creA* mutants showing that biosynthesis of arabinanases and L-arabinose catabolic enzymes is under control of CreA.**

INTRODUCTION

Carbon repression is a global regulatory mechanism by which in the presence of D-glucose or other rapidly metabolisable carbon sources the expression of genes involved in the utilisation of less-favoured carbon sources is repressed [19,27,29] for reviews on carbon repression in fungi). It allows microorganisms to cope fluently with changes in the carbon sources present in their environment. Genetic analysis has shown that carbon repression in *Aspergillus nidulans* is mediated by a major regulatory gene *creA*, which acts in a negative manner [2,3]. Mutations in *A. nidulans creA* result in derepression of a variety of activities, which are normally repressed by D-glucose [1,4,17]. *creA* mutations were isolated by several strategies including selection of pseudorevertants of *areA*-defective strains. The *creA* gene has been cloned and sequenced both from *A. nidulans* [7,8] and *A. niger* [9] and shown to encode a DNA-binding protein containing two zinc fingers of the Cys_2His_2 type, which are very similar to the zinc fingers of MIG1, the

repressor involved in D-glucose repression of the *GAL* and *SUC2* genes in *Saccharomyces cerevisiae* [24,25].

Plant cell walls consist of complex polysaccharides of which L-arabinan is one of the constituents. Hyphal fungi are able to degrade these polysaccharides into monomeric sugars which are subsequently metabolised further. The L-arabinan degrading system of *A. niger* consists of two distinct α-L-arabinofuranosidases (AbfA and AbfB) and an endo-1,5-α-arabinanase (AbnA) [32]. The expression of these three enzymes is under the control of pathway specific induction and carbon repression. Three arabinanase-encoding genes *abfA*, *abfB* and *abnA* have been cloned and characterised [11,12,13,14]. In the promoters of these three genes several putative CreA binding sites, i.e. sequence elements identical to the *A. nidulans* CreA-motif 5'-G/CPyGGPuG-3' [6,20], as well as some elements common to all arabinanase genes can be found [14].

Although the *A. niger creA* gene was cloned, the selection of *A. niger creA* mutants has thus far never been described. By conventional mutagenesis we have now isolated four *A. niger creA* mutants which are useful to establish whether various metabolic systems are under CreA control. As an example we have studied L-arabinan degradation and L-arabinose catabolism.

MATERIALS AND METHODS

***A. niger* strains, isolation of mutants and linkage analysis.** All strains used were descendants from *A. niger* N400 (CBS 120.49). N402 (*cspA1*) was used for CHEF analysis. NW141 (*areA1*, *bioA1*, *cspA1*, *pyrA13*) was isolated from NW140 (*areA1*, *bioA1*, *cspA1*)[15]. N616 [5] was used as a tester strain for genetic analysis.

UV-mutagenesis and isolation of *pyrA* mutants was performed as described by Goosen *et al*. [16]. The survival of the spores for the selection of the *pyrA13* marker was more than 50%. Selection of the *creA* mutants was done after UV-mutagenesis, resulting in 38% survival. Irradiated conidia ($4.5*10^7$) of strain NW141 were plated on 10 minimal medium (MM)[26] plates containing 1% (w/v) 4-aminobutanoic acid (GABA) and 1% (w/v) D-glucose. After 4 days of incubation at 30°C 80 colonies were picked and tested on various combinations of carbon and nitrogen sources.

Linkage analysis was performed as described by Bos *et al*. [5]. Tester strain N616 contains genetic markers on 6 linkage groups: *fwnA1* (I), *hisD4* (II), *lysA7* (III), *leuA1* (IV), *nicA1* (V) and *pabA1* (VI).

Medium and culture conditions. Mycelium was cultured on MM containing 0.02% (v/v) of a trace metal solution [36] and appropriate carbon and nitrogen sources. Where necessary media were supplemented with 4 µg biotin l^{-1}, 1 mg nicotinamide l^{-1}, 1.4 mg p-amino benzoic acid l^{-1}, 200 mg histidine l^{-1}, 200 mg leucine l^{-1}, 365 mg lysine l^{-1} and 1.22 g uridine l^{-1}. For plate tests MM was solidified with 1.5% (w/v) agar unless

stated otherwise. For expression studies strains were grown for 26 h on MM containing 10 mM ammonium tartrate, 0.05% (w/v) yeast extract and 2% (w/v) sucrose in an orbital shaker at 250 rpm at 30°C. Mycelium was then harvested, washed with MM, transferred to MM containing 10 mM ammonium tartrate, 1 % (w/v) carbon source as indicated and incubated for another 4 h.

Transformation of *A. niger*, northern analysis and CHEF analysis. Transformation of *A. niger* was essentially performed as described by Kusters-van Someren *et al*. [21] using the *A. niger pyrA* gene [16] as a selective marker. Plasmid pCRA006 [23] contains the *A. niger creA* and *pyrA* genes (the *A. niger creA* gene was isolated by heterologous hybridisation with a probe constructed from *A. nidulans creA*; the identity of the gene was established by comparing the restriction map of the cloned gene with the sequence of *A. niger creA* published by Drysdale *et al*. [9].

For northern analysis total RNA was isolated using TRIzol™ (Life Technologies) according to the manufacturers instructions. Total RNA (20 µg for each sample) was run on formaldehyde containing gels as described by Sambrook *et al.* [28] and transferred onto Hybond N (Amersham) membranes by capillary blotting in 10 X SSC. Hybridisation was done at 42°C in buffer containing 50% (w/v) formamide, 0.75 M NaCl, 50 mM sodium phosphate pH 7.4, 10 mM EDTA, 2 X Denhardt's, 0.1% (w/v) SDS and 10% (w/v) dextran sulphate. Northern blots were washed at 65°C down to 0.2 X SSC / 0.1% (w/v) SDS. Probes were prepared using the random priming method [28]. The following DNA fragments were used as probes: a 1.5 kb *Pst*I fragment from plasmid pC2X1 (containing the C-terminal region of *abfA*) [13], a 1.7 kb *Eco*RI-*Xho*I fragment from plasmid pB2 (containing the *abfB* full length cDNA) [11], a 1.1 kb *Eco*RI-*Xho*I fragment from plasmid pC2N4 (containing the *abn*A full length cDNA) [12] and a 0.9 kb *Eco*RI fragment from plasmid p28S (containing the *A. bisporus* gene encoding 28S rRNA) [30]. RNA levels were quantified by laser densitometric scanning of autoradiograms (Ultroscan XL, LKB).

Contour-clamped homogeneous electric field (CHEF) analysis was performed according to Verdoes *et al.* [35] with chromosomal DNA of *A. niger* N402. Hybridisation was performed using a 840 bp *Xho*1 - *Eco*R1 fragment of pCRA004 (containing an internal fragment of *creA*) [23] as a probe.

Preparation of cell extracts and enzyme assays. Preparation of cell extracts and assay of L-arabinose reductase and L-arabitol dehydrogenase were performed as described by Witteveen *et al*. [37]. Enzyme assays were performed on a COBAS Bio autoanalyser (Roche) connected to an MS-DOS computer for datalogging. Biochemicals were from Boehringer Mannheim. Protein concentration in extracts was determined as described previously [37] using the bicinchoninic acid method (Sigma). α-L-Arabinofuranosidase activity was determined by measuring hydrolysis of p-nitrophenyl-α-L-arabinofuranoside (pNP-A, Sigma) as described previously [32].

Western blotting. Denaturing electrophoresis in 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS was performed as described by Laemmli [22] in a Mini-V system (Life Technologies). Protein was blotted onto nitro-cellulose filters and blots were then incubated with specific antisera, followed by staining with alkaline phosphatase labelled goat anti-mouse IgG and alkaline phosphatase labelled goat anti-rabbit IgG as described by the manufacturer (BioRad). Antibodies raised against *A. niger* arabinofuranosidase A and B and endo-arabinanase A have been described previously [32].

Polyol extraction and determination. Extraction and determination of intracellular polyols was done as described by Witteveen *et al*. [37].

RESULTS

Isolation and characterisation of *A. niger creA*^{d} mutants

A. niger creA mutations were isolated as pseudorevertants of an *areA* loss-of-function mutation. Following UV mutagenesis of the *areA1* strain NW141, strongly growing colonies were selected on minimal medium containing 1% (w/v) D-glucose and 1% (w/v) 4-aminobutanoic acid (GABA). Preliminary growth tests on solid media containing different combinations of carbon and nitrogen sources revealed four putative *creA* mutants which were characterised in more detail. These four mutants were clearly derepressed for the use of GABA as a nitrogen source in the presence of D-glucose (Table 1). Three of the four mutants, *creA2*, *creA4* and *creA5*, also showed derepression for L-alanine and L-proline utilisation. Growth of the parental *areA* strain NW141 on GABA was very poor and comparable to growth on GABA + D-glucose. The same was observed for L-proline and L-proline + D-glucose. *A. nidulans areA* strains grow rather well on these amino acids, but growth is reduced when D-glucose is added due to repression by D-glucose of amino acid metabolism [29]. For *A. niger* this is only observed with L-alanine. The explanation for this behaviour is that GABA and L-proline, and to a lesser extent L-alanine, are poor carbon substrates for *A. niger*. In the presence of D-glucose the amino acids are only required as a nitrogen source, but as D-glucose represses amino acid metabolism, growth is still very poor.

Three classes of morphology were observed (Table 1). *creA5* exhibited a normal morphology, i.e. comparable to the parental *areA1* strain NW141. *creA2*, *creA4*, and to a lesser extent *creA1*, had difficulty to form conidiospores. The mycelium of *creA1* was yellow, unlike the parental strain and the other three mutants, which formed the usual white mycelium.

To test complementation of the mutations with the *A. niger creA* gene, the mutants were transformed with plasmid pCRA006, which contains the *A. niger creA* and *pyrA* genes. For all four mutants uridine prototrophic strains were obtained. Growth of these transformants on GABA/D-glucose was indistinguishable from the *areA pyrA*$^{+}$ strain NW140. In addition, whereas the morphology of *creA1*, *creA2* and *creA4* was clearly different from parental strain NW141, their *pyrA*$^{+}$ *creA*$^{+}$ transformants were reverted to NW140 morphology.

Table 1. Growth properties of *A. niger creA* mutants.
Growth characteristics were determined on plates as described under 'Methods' except that nitrate was omitted from the medium; 2.5 mM uridine was added; plates were solidified with agarose; 5 mM GABA, L-alanine or L-proline were used in the presence and absence of 1% (w/v) D-glucose. Morphology classes: normal, white mycelium and good sporulation; type A, yellow mycelium and moderate sporulation; type B, white mycelium and poor sporulation. Growth score: (+), very poor growth; (++), poor growth; (+++) moderate growth; (++++), good growth.

Strain	Relevant genotype	Morphology	Growth on:					
			GABA	GABA + D-glucose	L-proline	L-proline + D-glucose	L-alanine	L-alanine + D-glucose
NW141	*areA1*	Normal	+	+	+	+	++	+
NW142	*areA1 creA1*	Type A	++	++	+	+	++	+
NW143	*areA1 creA2*	Type B	++	++++	+	++	++	+++
NW145	*areA1 creA4*	Type B	++	++++	+	++	++	+++
NW146	*areA1 creA5*	Normal	+	+++	+	++++	++	++

The genetic localisation of the *creA1* (strain NW142) and *creA2* (strain NW143) mutations was determined by linkage analysis. From cross NW142//N616 a number of 58 out of 102 progeny carried *areA1* and those were tested for derepression of GABA utilisation in the presence of D-glucose. The recombination frequency between *creA1* and *leuA1* was 5.2%. All other markers gave recombination frequencies between 37% and 54%. Cross NW143//N616 gave comparable results. From a total progeny of 89, 33 recombinants contained *areA1*. Between *creA2* and *leuA1* 12.1% recombination was observed, while between *creA2* and the other markers recombination varied from 38% to 62%. This positioned *creA* on linkage group IV. Localisation of the *creA* gene on chromosome IV was confirmed by CHEF analysis (Fig. 1).

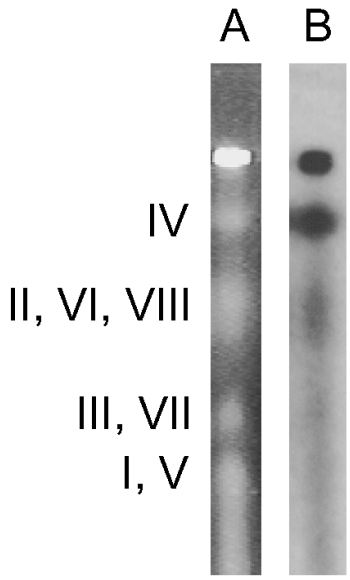
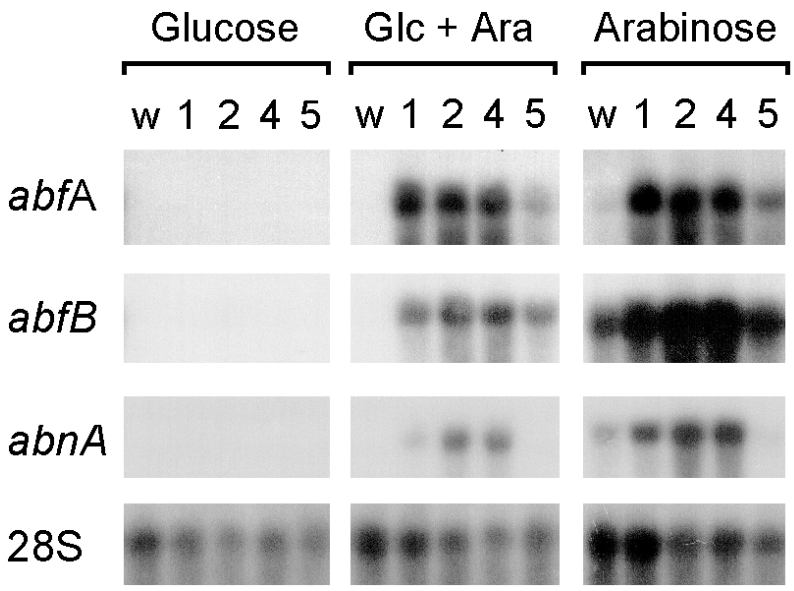


Fig. 1. Chromosome assignment of *creA* by CHEF analysis. Lane A, ethidium bromide stained chromosomes of wild-type *A. niger* strain N402 separated by CHEF electrophoresis. Lane B, hybridisation of the CHEF blot obtained from the gel shown in lane A with the 840 bp *Xho*1 - *Eco*R1 fragment of *creA*. Linkage groups of *A. niger* N402 are indicated by Roman numerals at the left.

Effects of *creA* mutations on expression of α-L-arabinofuranosidase and L-arabinose catabolic enzymes.

The possible involvement of CreA in repression by D-glucose of arabinanases and L-arabinose catabolic enzymes was studied in the *creA* mutants. Wild-type strain NW141 and *creA* mutants were grown for 26 h on minimal medium with 2% sucrose and mycelia were subsequently transferred to 1% L-arabinose, 1% L-arabinose + 1% D-glucose and 1% D-glucose for 4 h. Samples were taken to analyse arabinanase transcript levels, arabinanase

protein levels, activity of arabinanases and L-arabinose catabolic enzymes and accumulation of the intermediates of L-arabinose metabolism, L-arabitol and xylitol.



	Glc + Ara					Arabinose				
	W	1	2	4	5	W	1	2	4	5
abfA	0	8.8	14.9	11.9	4.2	1	8.1	10.5	9.5	6.4
abfB	0	0.9	1.5	2.0	1.0	1	1.3	3.1	2.6	2.1
abnA	0	0.5	3.5	2.7	0.2	1	2.6	5.0	4.5	0.3

Fig. 2. Arabinanase expression in *A. niger* wild-type and *creA* mutants. Mycelium was grown on sucrose, washed and transferred to D-glucose, D-glucose + L-arabinose (Glc + Ara) or L-arabinose as indicated in the figure. Northern blots were prepared as described under Methods and hybridised separately with *abfA*, *abfB*, *abnA* and 28S probes. RNA levels were quantified by laser densitometric scanning of autoradiograms. The 28S rRNA probe was used as an internal control. In the Table the amounts of *abfA*, *abfB* and *abnA* mRNA relative to 28S rRNA are given for the growth conditions L-arabinose and D-glucose + L-arabinose. After growth on D-glucose transcript levels were insignificant. In addition, for each of the three genes values were normalised to the induced wild-type level (i.e. NW141 grown on L-arabinose) which was set to 1. **W**, wild-type; **1**, *creA1*; **2**, *creA2*; **4**, *creA4*; **5**, *creA5*.

Northern analysis using probes of *abfA*, *abfB* and *abnA* revealed that upon transfer to L-arabinose the wild-type strain expressed all three arabinanases (Fig. 2). Upon transfer of the wild-type strain to D-glucose or L-arabinose + D-glucose no expression of the three arabinanase genes was detected. In the *creA* mutants, however, the arabinanase genes were clearly expressed on L-arabinose + D-glucose. The strongest derepression was observed for mutants *creA2* and *creA4*, whereas *creA1* and *creA5* resulted in moderate derepression. On L-arabinose a higher expression level of the arabinanase genes was observed for all four

creA mutants except for *abnA* in mutant *creA5*. Again the effect was most pronounced in the case of *creA2* and *creA4*. Relative to the induced wild-type levels, *abfA* and *abnA* expression was increased more than *abfB*.

Arabinanase activities were measured in the culture filtrates. During growth on L-arabinose + D-glucose α-L-arabinofuranosidase (Abf) activity, measured as pNP-A hydrolysis, was insignificant in a culture filtrate of the wild-type strain, but clearly present in the *creA* mutants (Fig. 3). Abf activity of strain *creA2* and *creA4* even approached the induced wild-type level (i.e. comparable to NW141 grown on L-arabinose). On L-arabinose Abf activity in *creA2*, *creA4* and *creA5* was 1.6-, 2.5- and 2.1-fold the wild-type level respectively, whereas *creA1* was comparable to wild-type. Endo-arabinanase activity remained too low to be accurately measured.

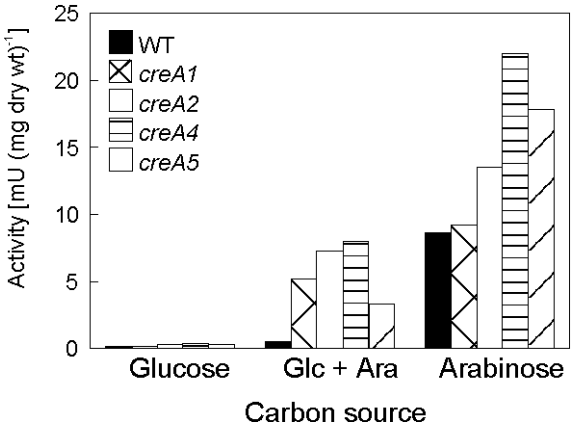


Fig. 3. α-L-arabinofuranosidase activity in culture filtrates of *A. niger* wild-type and *creA* mutants. Growth was performed as described in the legend of Fig. 2. Arabinofuranosidase activity was determined by measuring hydrolysis of pNP-A. Activities are expressed as mU (mg dry weight)$^{-1}$ (equivalent to nmol pNP produced min^{-1} (mg dry weight)$^{-1}$). DW, dry weight; WT, wild-type. Data are the average of two experiments.

Immunochemical detection of the arabinanase proteins by western blotting qualitatively corroborated the results of the northern analysis and the activity measurements (data not shown) and confirmed that of the two α-L-arabinofuranosidases present in wild-type *A. niger* (AbfA and AbfB), AbfB is the main activity [33]. Endo-arabinanase was not detected. Both AbfA and AbfB were clearly derepressed in the *creA* mutants grown on L-arabinose + D-glucose. On L-arabinose, the quantity of AbfB produced by the *creA* mutants was apparently comparable to that observed for the wild-type strain, but the AbfA levels of the *creA* mutants were higher than that of the wild-type. However, one should realise that western analysis does not provide quantitative data.

Activities of the enzymes involved in catabolism of L-arabinose (L-arabinose reductase, L-arabitol dehydrogenase, L-xylulose reductase and xylitol dehydrogenase) were analysed in parallel. For all strains the activity of L-arabinose reductase and L-arabitol dehydrogenase was low during growth on D-glucose (Fig. 4). On D-glucose + L-arabinose

the L-arabinose reductase and L-arabitol dehydrogenase activities were still low in wild-type strain NW141, but were increased in the *creA* mutants. Similar results were obtained for L-xylulose reductase and xylitol dehydrogenase (data not shown). Derepression was most pronounced in the case of L-arabitol dehydrogenase for which the activities in *creA1* and *creA4* were approximately 4-fold the wild-type activity. In contrast to the results obtained for arabinanases where *creA2* and *creA4* were derepressed strongest, *creA1* and *creA4* were derepressed most for the L-arabinose catabolic enzymes. During growth on L-arabinose the activities of L-arabinose reductase and L-arabitol dehydrogenase were up to 2-fold higher in the *creA* mutants than in wild-type strain NW141.

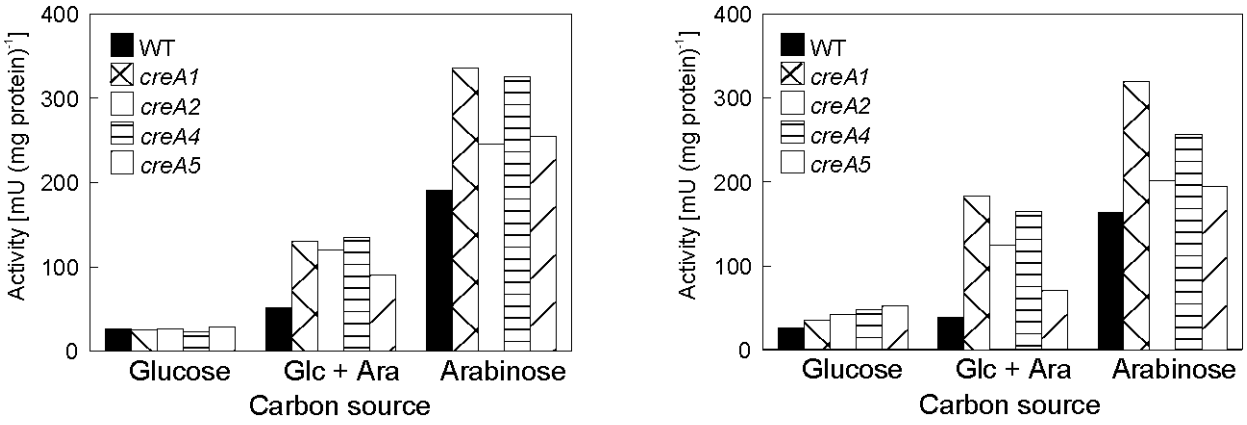


Fig. 4. Specific activity of L-arabinose reductase and L-arabitol dehydrogenase in cell extracts of *A. niger* wild-type and *creA* mutants. Mycelium was cultured as described in the legend of Fig. 2. Activities were measured as described under 'Methods'. WT, wild-type. Data are the average of two experiments.

Derepression of the L-arabinose catabolic enzymes during growth on a combination of D-glucose and L-arabinose might result in intracellular accumulation of the polyol compounds xylitol and L-arabitol, which are intermediates in L-arabinose degradation [37]. L-arabitol levels cannot be determined unequivocally due to simultaneous formation of D-arabitol from the pentose phosphate pathway intermediate D-xylulose-5-phosphate under all conditions and the inability to distinguish between D- and L-arabitol by HPLC analysis. The appearance of xylitol, however, is diagnostic for the presence of L-arabitol. No xylitol was observed in any of the strains during growth on D-glucose, whereas a considerable amount of xylitol accumulated during growth on L-arabinose (Table 2). On D-glucose + L-arabinose, a very low level of xylitol was observed in the wild-type strain, but xylitol did accumulate in the *creA* mutants indicative of derepression of L-arabinose catabolism. As expected, arabitol was found under all growth conditions, but the levels were relatively high during growth on L-arabinose, implying accumulation of L-arabitol.

Table 2. Intracellular polyol concentrations in *A. niger* wild-type and *creA* mutants during growth on D-glucose, D-glucose + L-arabinose and L-arabinose. Polyol concentrations are expressed in μmol (g dry wt)$^{-1}$. Data are the mean of two experiments.

Strain	Relevant genotype	D-glucose		D-glucose + L-arabinose		L-arabinose	
		Xylitol	Arabitol	Xylitol	Arabitol	Xylitol	Arabitol
NW141	Wild-type	0	16	1	19	*25*	106
NW142	*creA1*	0	7	19	25	*30*	100
NW143	*creA2*	0	40	13	59	*56*	136
NW145	*creA4*	0	38	14	58	66	154
NW146	*creA5*	0	14	6	26	23	93

DISCUSSION

Hyphal fungi seem to share the regulatory system responsible for carbon repression which was first described for *A. nidulans* on the basis of mutations in *creA* [1,4,17] and further substantiated by the cloning of this gene [7,8]. The *A. niger creA* gene was found to be very similar to the *A. nidulans creA* gene and, moreover, was shown to be functional in *A. nidulans* [9]. However, thus far no *creA* mutants have been described for *A. niger*. In this report we describe such mutants which have, as expected, a derepressed phenotype. The following results imply that the mutants we isolated are impaired in *creA*. The mutants were derepressed for a number of systems involved in carbon catabolism. In an *areA* background the *creA* mutations allowed utilisation of GABA, L-proline or L-alanine as nitrogen sources in the presence of D-glucose (Table 1). Whereas the parent showed repression of arabinanases and L-arabinose catabolism in medium containing both L-arabinose and D-glucose, these functions were clearly derepressed in the *creA* mutants under the same conditions (Figs. 2 to 4). Finally, transformation of the mutant strains with the *A. niger creA* gene resulted in a phenotype that was indistinguishable from the parental *areA* strain. The finding that the *A. niger creA* gene was able to complement the mutations is a strong indication that we have isolated *creA* mutants.

The different *A. niger creA* alleles also displayed non-hierarchical heterogeneity, observed with *A. nidulans creA* mutants as well [3] and indicative for a direct effect of CreA on transcription. For example, whereas *creA2* and *creA4* were strongly derepressed for the use of GABA and L-alanine in the presence of D-glucose and less for L-proline, *creA5* showed exactly the opposite phenotype.

A. niger creA mutants further exhibited decreased growth rates and reduced sporulation. In addition, *A. niger creA1* produced a yellow pigment, which is probably a secondary metabolite whose biosynthesis is normally repressed by D-glucose.

From our results it appears that *creA2* and *creA4* are the most severe alleles. These alleles are extremely useful to investigate the involvement of CreA in control of other systems subject to carbon repression. Cloning and sequencing of the *creA* alleles may be useful to identify domains in the protein, other than the zinc-finger region, that are important for its function. This was recently done by Shroff *et al.* [31] for a number of *A. nidulans creA* alleles. Three of the *A. nidulans creA* alleles analysed have missense mutations in the zinc finger domain whereas four other mutations result in truncations of CreA between the zinc finger domain and the C-terminus of the protein.

Analysis of arabinanase expression in *A. nidulans creA* mutants has demonstrated that the arabinanase system is suitable to investigate carbon repression in this fungus [34]. L-arabinose and L-arabitol induce the *A. niger* arabinanases (AbfA, AbfB and AbnA), whereas addition of D-glucose prevents this induction [33,34]. Similarly, enzymes involved in catabolism of L-arabinose are expressed during growth on L-arabinose, but not on D-glucose [37]. These observations suggest repression of arabinanases and L-arabinose catabolic enzymes by D-glucose. In this report we show that this repression is in fact mediated by CreA. On a combination of L-arabinose + D-glucose no expression of *abfA*, *abfB* and *abnA* was detected in the wild-type strain. In the *creA* mutants, however, the three arabinanase genes were clearly expressed under these conditions (Fig. 2). Biosynthesis and secretion of the arabinanases was confirmed by western analysis and activity in the case of AbfA and AbfB, but not for AbnA (Fig. 3). The absence of endo-arabinanase protein is explained by the delayed expression of *abnA* compared to *abfA* and *abfB*, which has been observed previously [14]. L-arabinose reductase and L-arabitol dehydrogenase, the enzymes involved in L-arabinose catabolism, were also derepressed in the *creA* mutants (Fig. 4). Several putative CreA binding sites are present in the promoters of the three arabinanase-encoding genes [14] and it is most likely that CreA directly represses the genes encoding arabinanases and L-arabinose catabolic enzymes by binding to its cognate sequence(s) in the promoters of these genes. This is substantiated by the non-hierarchical heterogeneity amongst the different *creA* alleles. Thus, while *creA2* and *creA4* showed stronger derepression of *abnA* than of *abfB*, derepression of *abnA* was much less than that of *abfB* in *creA5*. Similarly, derepression of arabinanase genes was more pronounced for *creA2* and *creA4* than for *creA1*, but *creA1* was more derepressed for L-arabinose reductase and L-arabitol dehydrogenase. Two other possible mechanisms of repression of genes encoding arabinanases and L-arabinose catabolic enzymes are (1) a cascade mechanism, *i.e.* repression of a common transcription activator protein and (2) lack of inducer formation. A common transcription activator for arabinanases has been proposed by Flipphi *et al.* [14] on the basis of the finding that extra gene copies of either *abfA* or *abfB* decreased expression of the other *abf* gene and, more clearly, of the more weakly expressed *abnA* gene. A cascade mechanism for repression is operating in the case of the *alc* system in *A. nidulans*. The gene encoding the transcription activator of the *alc* system, *alcR*, is repressed by D-glucose, partially preventing induction of the *alc* system [20]. However, most of the *alc* genes, including *alcA* which is the structural gene for alcohol dehydrogenase I, are also repressed directly by CreA [10,20]. The second

alternative for direct repression is lack of inducer formation. Arabinanases are induced by L-arabitol, an intermediate of L-arabinose metabolism [33], and derepression of L-arabinose uptake and L-arabinose reductase, which could result in intracellular accumulation of L-arabitol, might be sufficient to induce expression of arabinanases. Such a mechanism operates for example in the case of the *gal* genes in *S. cerevisiae*, where MIG1 represses the expression of the D-galactose permease thereby reducing the level of functional inducer [18]. Proper investigation of the relative contribution of the three repression mechanisms mentioned requires isolation of the genes encoding the putative arabinanase transcription activator, the L-arabinose permease and the enzymes involved in L-arabinose catabolism.

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Chapter 3

The *abfB* gene encoding the major α-L-arabinofuranosidase of *Aspergillus nidulans*: Nucleotide sequence, regulation and construction of a disrupted strain.

M.M.C. Gielkens, L. González-Candelas, P. Sánchez-Torres, P.J.I. van de Vondervoort, L. H. de Graaff, J. Visser

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ABSTRACT

Using a DNA fragment containing the *Aspergillus niger abfB* gene as a probe the homologous *Aspergillus nidulans* gene, designated *abfB*, has been cloned from a genomic library containing size-selected *Hind*III fragments. The nucleotide sequence of the *abfB* gene shows strong homology with the *A. niger abfB*, *Trichoderma reesei abf-1* and *Trichoderma koningii* α-L-arabinofuranosidase / β-xylosidase genes. Regulation of *abfB* expression has been investigated in cultures induced with L-arabitol. The accumulation of *abfB* mRNA, total α-L-arabinofuranosidase activity and AbfB protein levels have been determined in a wild-type *A. nidulans* strain as well as in different mutant strains. These strains are affected either in their response to ambient pH (*palA1* and *pacC*^{c}*14* mutants), carbon catabolite repression (*creA*^{d}*4* mutant), the ability to utilise L-arabitol as a carbon source (*araA1* mutant) or a combination of both latter mutations (*araA1 creA*^{d}*4*). The results obtained indicate that the expression of the *A. nidulans abfB* gene was higher at acidic pH and was super-induced in this double mutant. Furthermore, disruption of the *abfB* gene demonstrated that in *A. nidulans* AbfB is the major *p*-nitrophenyl-α-L-arabinofuranoside-hydrolysing activity but at least one minor activity is expressed, which is involved in the release of L-arabinose from polysaccharides.

INTRODUCTION

L-arabinose is a constituent of plant cell wall polysaccharides. It is found in a polymeric form as in L-arabinan, in which the backbone is formed by α-1,5-linked L-arabinose residues which can be branched via α-1,2- and α-1,3-linked L-arabinofuranose side chains. L-arabinose is also found as a side chain residue in arabinogalactans (α-1,3- or α-1,6-linked), arabinoxylans (α-1,2- or α-1,3-linked) and in pectin (α-1,3-linked). The enzymatic modification of these polysaccharides is technologically relevant in the processing of agricultural products like fruits, vegetables and cereals [32,33].

In nature different microorganisms secrete endo-arabinanases and α-L-arabinofuranosidases (Abf, EC 3.2.1.55) to degrade polysaccharides containing L-

arabinose. Most commercial enzyme preparations containing L-arabinanases are obtained from filamentous fungi like *Aspergillus niger*. This fungus secretes two arabinofuranosidases (AbfA and AbfB) and one endo-arabinanase, which all three have been purified and characterised. AbfA acts only on small linear α-1,5-linked L-arabinofuranosyl oligosaccharides, whereas AbfB hydrolyses 1,5-, 1,3- and 1,2-α-linkages in both oligosaccharides and in polysaccharides, which contain terminal non-reducing L-arabinofuranoses in side chains [15,22,28]. The encoding genes have been cloned and characterised [5,6,7,8]. In *A. niger* the synthesis of these enzymes is induced by both L-arabinose, the final product of L-arabinan degradation, and by L-arabitol, an intermediate of the catabolic pathway of L-arabinose, though the latter is a stronger inducer [29]. Furthermore, the expression of the L-arabinanase system is under carbon catabolite repression when more preferable carbon sources like D-glucose are present in the medium [23,29].

Like in *A. niger*, L-arabinanase biosynthesis in *A. nidulans* is induced by L-arabinose-containing substrates as e.g. sugar beet pulp, by the monomeric sugar L-arabinose and more strongly by L-arabitol [21]. When correlating arabinanase biosynthesis and the accumulation of L-arabitol in a L-arabitol dehydrogenase negative mutant, de Vries *et al.* [34] obtained evidence that L-arabitol is the actual inducer. Besides an endo-arabinanase activity, only one α-L-arabinofuranosidase (Abf) activity has been found in *A. nidulans*. These two proteins show immunological cross-reactivity with antibodies raised against *A. niger* AbnA and AbfB, respectively, and these enzymes are also similar to the *A. niger* counterparts in their kinetic and physico-chemical properties [21]. Van der Veen *et al.* [30] investigated the regulation of these two extracellular enzyme activities and of the enzymes of the L-arabinose catabolic pathway generating the inducer, in particular with respect to carbon catabolite repression. In order to extend the previous analysis to the transcriptional level we have now cloned and characterised the *A. nidulans abfB* gene. Furthermore, disruption of the *abf*B gene will facilitate us to identify possible new minor Abf functions.

MATERIALS AND METHODS

Strains, media and culture conditions. *Escherichia coli* DH5α was used as a host for cloning experiments. All *Aspergillus* strains used in this paper are described in Table 1. See Clutterbuck [2] for

definition of gene symbols. *Aspergillus* minimal medium was prepared as described by Pontecorvo *et al*. [19] and contained per litre 0.04 ml of a trace element solution [31]. Strains used in the shift experiment were pre-grown for 18 h at 37 °C in 250 ml minimal medium (MM) containing 100 mM D-glucose and 0.05 % (w/v) yeast extract. After harvesting, the mycelium was washed with 0.9 % (w/v) NaCl and 3 g portions were transferred to 50 ml MM containing 50 mM L-arabitol or 50 mM L-arabitol and 20 mM glycerol. These transfer cultures were grown for additional periods of 6 and 10 h. Where necessary the media were supplemented with 1.5 mg l^{-1} p-aminobenzoate or 4 µg l^{-1} biotin.

DNA isolation and manipulations. Fungal DNA was obtained as described earlier [20]. Amplification of the *A. niger abfB* gene was done as described previously [25] using oligonucleotides Abf2 (5'-GACCTATTTACAAAGCTTTCTCC-3'), which anneals 85 bp downstream the stop codon, and Abf4 (5'-GAGCCTGCAGTAATGCTCCACAATGTTCTCC-3'), which includes the ATG translation start codon.

Construction and screening of an *Aspergillus nidulans* partial genomic library. Southern blot analyses of *A. nidulans* DNA using a 1.6 kb DNA fragment obtained by PCR representing the entire *A. niger abfB* gene as a probe were carried out under different hybridisation and washing conditions. This revealed the existence of multiple hybridising DNA fragments. To avoid isolating false positives upon screening library the following scheme was devised. *A. nidulans* DNA was digested with *Hin*dIII. Samples were fractionated in triplicate by agarose gel electrophoresis. After transfer and UV fixation of the DNA to a nylon membrane, the membrane was cut into three pieces containing the same samples.

Table 1. *Aspergillus* strains used in this article

Strain	Genotype	Source/reference
A. niger		
N402	*cspA1*	Derived from CBS 120.49
A. nidulans		
V023	*argB2*, *biA1*, *metG1*	M. A. Peñalva*
WG096	*pabaA1*, *yA2*	FGSC 187
G094	*araA1*, *biA1*, *wA2*	[3]
creA^{d}4	*biA1*, *creA^{d}4*	H. N. Arst, Jr †
NW186	*araA1*, *biA1*, *creA^{d}4*, *cnxH4*	This study
pacC^{c}14	*biA1*, *pacC^{c}14*	H. N. Arst, Jr †
palA1	*pabaA1*, *palA1*	H. N. Arst, Jr †
NW187	*biA1*, *creA^{d}4*, *pyrG90*	This study
NW190	*biA1*, *creA^{d}4*, *pyrG90*, Δ*abfB-pyrA*$^{+}$	This study

* CIB, CSIC, Madrid.

† Royal Postgraduate Medical School, London.

Each one was hybridised under heterologous conditions (last wash step with 4 × SSC and 0.1 % (m/v) SDS at 60 °C) with either the complete *A. niger abfB* gene, a 0.9 kb *Kpn*I fragment containing the 5´ region of the *A. niger abfB* gene, or the remaining 0.7 kb fragment which corresponds to the 3´ region of the *A. niger abfB* gene. Comparison of the hybridisation patterns showed that a 6 kb *Hin*dIII fragment hybridised with the three probes under heterologous conditions. Subsequently, a partial library of *A. nidulans* was constructed. *A. nidulans* DNA was completely digested with *Hin*dIII and DNA fragments were separated through a 0.7 % (m/v) agarose gel electrophoresis. Fragments between 5 and 7 kb in length were recovered from the gel using a Geneclean kit (Bio 101 Inc.) and ligated into pBluescript II SK (+) which had previously been digested with *Hin*dIII and dephosphorylated. The ligated fragments were used to transform *E. coli* DH5α competent cells. The library was screened by colony hybridisation under heterologous conditions using the PCR amplified *A. niger abfB* gene as a probe. Approximately 12,000 recombinant clones were screened.

Sequence determination and analysis. DNA was sequenced by the dideoxynucleotide chain termination method [on double stranded plasmid (Sequenase 2.0, Amersham). A series of nested deletions were obtained by the exoIII-S1 nuclease method [12]. In addition, synthetic oligonucleotides were used to determine the sequence on both DNA strands. Computer analysis was done using the PC/GENE program (IntelliGenetics) and version 7 of the Genetics Computer Group package (GCG, Madison). The DNA sequence of the *A. nidulans abfB* gene has been deposited in the EMBL database under accession number Y13759.

RNA isolation and northern analysis. Total RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's instructions. Northern analysis was performed as described by Sambrook *et al.* [24]. Ten μg of total RNA was applied per lane. Northern blots were probed with the 0.65 kb *Eco*RI-*Kpn*I *Aspergillus nidulans abfB* fragment or with the 0.9 kb *Eco*RI fragment from the *Agaricus bisporus* 28S rDNA gene [27], which was used as an internal control. The blots were washed down to 0.2 x SSC at 65 °C. RNA levels were quantified by liquid scintillation analysis in a Packard Ultracarb 1500. Samples were corrected for loading differences using the 28S rDNA. All values were normalised for the sample of WG096 transferred to L-arabitol and grown for 10 h.

Construction of an *abfB* disruption plasmid. Plasmid pH12S1 containing the 3.6 kb *Sma*I insert of the *abfB* gene was digested using *Xba*I and *Hin*dIII and ligated into pGEM7, resulting in plasmid pLIG318. *Sal*I-digested pLIG318 was ligated with a 2.4 kb *Xho*I fragment containing the *A. niger pyrA* gene (EMBL Acc. No. X06626), generating pLIG343. In this plasmid 400 bp of the *abfB* coding region was replaced by the selection marker *pyrA*. The *Bam*HI site located in the *pyrA* gene was modified to a *Xho*I site using a *Bam*HI-*Xho*I linker (5'-GATCACTCGAGT-3') which retains the correct reading frame. The resulting plasmid pIM3005 was digested using *Bam*HI and the 5.6 kb *abfB* disruption fragment was used for transformation.

Determination of α-L-arabinofuranosidase (Abf) activity and western blotting. α-L-arabinofuranosidase activities were measured at 37 ºC using *p*-nitrophenyl-α-L-arabinofuranoside (pNP-A) as a substrate [28]. Western blotting was also performed as described by van der Veen *et al.* [28]. The Abf detection by enzyme staining was conducted as described by Gallego *et al.* [9] using 4-methylumbelliferryl-α-L-arabinofuranoside (MU-ara) in 50 mM sodium acetate buffer pH 4.0 to visualise Abf activities.

RESULTS AND DISCUSSION

Cloning of the *A. nidulans abfB* gene.

Southern blot analyses of *A. nidulans* DNA revealed the existence of multiple hybridising DNA fragments when probed with a PCR fragment representing the entire *A. niger abfB* gene. A strategy was devised to avoid the isolation of false positives upon screening of a library (see Methods). Twenty-two hybridising colonies were found when a partial plasmid library was screened. Physical maps of the plasmids isolated from these colonies allowed us to group them into five classes. One member of each class was further analysed by Southern blot analysis using either the 5' or 3' region of the *A. niger abfB* as probes under heterologous conditions. One of these plasmids, designated pH12, showed strong hybridisation signals with both probes in an overlapping region, thus being likely to contain a gene homologous to *A. niger abfB*. A 3.6 kb *Sma*I fragment from plasmid pH12, shown in Fig. 1, was subcloned in both orientations into the *Eco*RV site of pBluescript, yielding plasmids pH12S1 and pH12S4. It is interesting to note that of the twenty two positive clones initially isolated, six of those, all belonging to the same class, contained the *abfB* gene. The remaining clones were not further analysed since they might represent false positives.

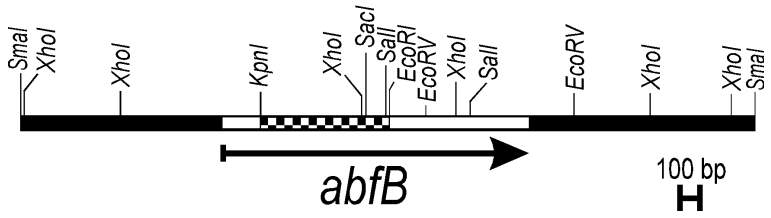

Fig. 1. Restriction map of the *Sma*I fragment containing the *A. nidulans abf*B gene. The *Kpn*I-*Eco*RI DNA fragment which hybridises with probes derived from both 5'and 3'regions of the *A. niger abf*B gene is indicated by a boxed pattern.

Sequence analysis of the *A. nidulans abfB* gene.

The nucleotide sequence of a 3141 bp *Sma*I-*Xho*I fragment of pH12 was determined for both strands. The determined sequence contains an open reading frame of 1530 bp. The predicted amino acid sequence of the encoding protein consists of 510 amino acids and contains a putative signal peptide of 24 amino acids. The mature protein has a calculated molecular mass of 50.6 kDa and a calculated pI of 3.9.

```
ABFBNID  MTMSRSSRSSVLALALATGSLVAAGPCDIYSSGGTPCIAAHSTTRALYSSYNGPLYQVQR
ABFBNIG  MF----SRRNLVALGLAAT--VSAGPCDIYEAGDTPCVAAHSTTRALYSSFSGALYQLQR
ABF1REE  ML---SNARIIAAGCIAAGSLVAAGPCDIYSSGGTPCVAAHSTTRALFSAYTGPLYQVKR
XYL1KON  ML---SNARIIAAGCIAAGSLVAAGPCDIYSSGGTPCVAAHSTTRALFSAYTGPLYQVKR
         *           *   *    * *******  * *** ********* *   * ***  *

ABFBNID  ASDGTTTTITPLSAGGVADASAQDAFCENTTCLITIIYDQSGNGNDLTQAPPGGFNGPDV
ABFBNIG  GSDDTTTTISPLTAGGVADASAQDTFCANTTCLITIIYDQSGNGNHLTQAPPGGFDGPDV
ABF1REE  GSDGATTAISPLSSG-VANAAAQDAFCAGTTCLITIIYDQSGRGNHLTQAPPGGFSGPES
XYL1KON  GSDGATTAISPLSSG-VANAAAQDAFCAGTTCLITIIYDQSGRGNHLREAPPGGFSGPES
          **  ** * **  * ** * *** **  ************* ** *  ****** **

ABFBNID  GGYDNLAGAIGAPVTLNGKKAYGVFVSPGTGYRNNEAIGTATGDEPEGMYAVLDGTHYND
ABFBNIG  DGYDNLASAIGAPVTLNGQKAYGVFMSPGTGYRNNEATGTATGDEPEGMYAVLDGTHYND
ABF1REE  NGYDNLASAIGAPVTLNGQKAYGVFVSPGTGYRNNAASGTAKGDAAEGMYAVLDGTHYNG
XYL1KON  NGYDNLASAIGAPVTLNGQKAYGVFVSPGTGYRNNAASGTAKGDAAEGMYAVLDGTHYNG
          ****** ********** ****** ********* * *** **  *************

ABFBNID  GCCFDYGNAETSSLDTGNGHMEAIYYGTNTAWGYGAGNGPWIMADLENGLFSGQSSDYNA
ABFBNIG  ACCFDYGNAETSSTDTGAGHMEAIYLGNSTTWGYGAGDGPWIMVDMENNLFSGADEGYNS
ABF1REE  ACCFDYGNAETNSRDTGNGHMEAIYFGDSTVWGTGSGKGPWIMADLENGLFSGSSPGNNA
XYL1KON  ACCFDYGNAETNSRDTGNGHMEAIYFGDSTVWGTGSGKGPWIMADLENGLFSGSSPGNNA
          ********** * *** ******* *  * ** * * ***** * ** ****     *

ABFBNID  GDPSISYRFVTAILKGGPNLWALRGGNAASGSLSTYYNGIRPTDASGYNPMSKEGAIILG
ABFBNIG  GDPSISYSFVTAAVKGGADKWAIRGGNAASGSLSTYYSGARP-DYSGYNPMSKEGAIILG
ABF1REE  GDPSISYRFVTAAIKGQPNQWAIRGGNAASGSLSTFYSGARP-QVSGYNPMSKEGAIILG
XYL1KON  GDPSISYRFVTAAIKGQPNQWAIRGGNAASGSLSTFYSGARP-QVSGYNPMSKEGAIILG
         ******* ****  **    ** ************ * * **   ***************

ABFBNID  IGGDNSVSAQGTFYEGAMTDGYPDDATENSVQADIVAAKYATTSLISGPALTVGDTVSLK
ABFBNIG  IGGDNSNGAQGTFYEGVMTSGYPSDDVENSVQENIVAAKYVSGSLVSGPSFTSGEVVSLR
ABF1REE  IGGDNSNGAQGTFYEGVMTSGYPSDATENSVQANIVAARYAVAPLTSGPALTVGSSISLR
XYL1KON  IGGDNSNGGQGTFYEGVMTSGYPSDATENSVQANIVAARYAVAPLTSGPALTVGSSISLR
         ******   ******* ** *** *  *****  **** *    * ***  * *   **

ABFBNID  VTTSGYDTRYIAHTGSTINTQVVSSSSSSTLKQQASWTVRTGLASTAAANGCVSFESVDT
ABFBNIG  VTTPGYTTRYIAHTDTTVNTQVVDDDSSTTLKEEASWTVVTGLANSQ----CFSFESVDT
ABF1REE  ATTACCTTRYIAHSGSTVNTQVVSSSSATALKQQASWTVRAGLAN----NACFSFESRDT
XYL1KON  ATTACCTTRYIAHSGSTVNTQVVSSSSATALKQQASWTVRAGLAN----NACFSFESQDT
          **    ******   * *****   *   **  *****  ***       * **** **

ABFBNID  PGSYIRHSNFALLLNANDGTKLFSEDATFCPQDSFNDDGTNSIRSWNYPTRYWRHYENVL
ABFBNIG  PGSYIRHYNFELLLNANDGTKQFHEDATFCPQAPLNGEGT-SLRSWSYPTRYFRHYENVL
ABF1REE  SGSYIRHSNFGLVLNANDGSKLFAEDATFCTQAGINGQGS-SIRSWSYPTRYFRHYNNTL
XYL1KON  SGSYIRHSNFGLVLNANDGSKLFAEDATFCTQAGINGQGS-SIRSWSYPTRYFRHYNNTL
          ****** ** * ****** * * ****** *   *  *  * *** ***** *** * *

ABFBNID  YVASNGGVNTFDAATAFTDDVSWVVADGFA-
ABFBNIG  YAASNGGVQTFDSKTSFNNDVSFEIETAFAS
ABF1REE  YIASNGGVHVFDATAAFNDDVSFVVSGGFA-
XYL1KON  YIASNGGVHVFDATAAFNDDVSFVVSGGFA-
         * ******  **    *  ***      **
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Fig. 2. Amino acid sequence comparison of *A. nidulans* AbfB (ABFBNID), *A. niger* AbfB (ABFBNIG) [5], *T. reesei* Abf1 (ABF1REE) [18] and *T. koningii* Xyl1 (XYLKON) [14] sequences. Alignment was done with the CLUSTAL V program. Identical amino acids (*) are shown.

Similarity searches against databases revealed that *A. nidulans* AbfB is homologous to *A. niger* N400 AbfB (77% identity) [5], and also to *Trichoderma reesei* Abf1 [18] and to the *Trichoderma* Abf/β-xylosidase [14] proteins. Alignment of *A. nidulans* AbfB with these other fungal proteins, excluding putative signal peptides, shows a high overall similarity which is calculated to be 64% (Fig. 2).

A. niger AbfB and *T. koningii* Abf/β-xylosidase proteins have recently been included in a new family of glycosyl hydrolases (Family 54) [13]. Family 54 thus includes *A. nidulans* AbfB as well. It is interesting to note that, whereas both *Trichoderma* enzymes also show β-xylosidase activity, this was not found for the *A. niger* equivalents [5] or for *A. nidulans* AbfB.

In the promoter region of the *A. nidulans abf*B gene, consensus recognition sequences were found for the wide domain regulators CreA (-127, -253 and -435) and PacC (-299, -312, -416, -742, -884, -973 and -985), that mediate carbon catabolite repression and pH regulation, respectively.

Regulation of *abfB* expression.

Van der Veen *et al.* [30] studied the role of the CreA repressor protein in the expression of several enzymes of the L-arabinose catabolic pathway and in the biosynthesis of AbfB in *A. nidulans*. In the *creA*^{d}*4* and *creA*^{d}*30* strains, very marked, elevated inducibility was noticed both for the extracellular enzyme and for the intracellular enzymes. De Vries *et al.* [34] characterised the *araA1* mutant that turned out to lack NAD^{+}-dependent L-arabitol dehydrogenase activity and is unable to utilise L-arabinose or L-arabitol. This strain featured elevated expression of AbfB caused by the accumulation of L-arabitol. Here we have extended these studies by comparing under inducing conditions only the expression of *abfB* in the wild-type strain, the *araA1* mutant, a carbon catabolite derepressed mutant (*creA*^{d}*4*) [1] and an *araA1 creA*^{d}*4* double mutant. The strains were cultivated as described in Methods. In this way, any biosynthesis of AbfB can be addressed to induction by L-arabitol, since pre-growth on D-glucose strongly represses AbfB expression and therefore no AbfB is present at the start of the induction period.

We first determined the pNP-A hydrolysing activities in the culture filtrates in the various *A. nidulans* strains, which is shown in Fig. 3. Hydrolysis of pNP-A was significantly increased in the *creA*^{d} mutant compared to those measured in the wild-type.

The increase of pNPase activity was less in the *araA* mutant than in the *creA*^{d} mutant. In the *araA1 creA*^{d}*4* double mutant, however, the level of pNP-A hydrolysing activity was higher compared to those determined in the two single-mutant strains separately. In this strain, the combination of the *creA*^{d} mutation and the intracellular accumulation of inducer leads to super-induction.

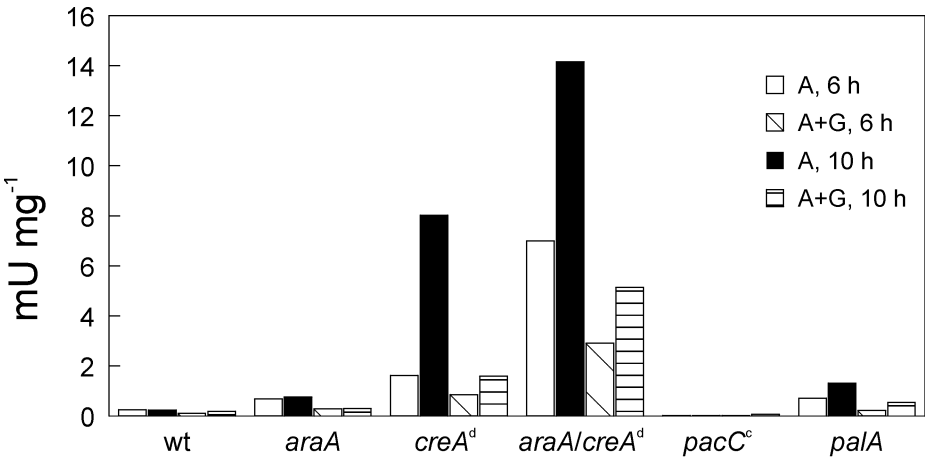

Fig. 3. Determination of pNPase activities in the culture filtrates of various *A. nidulans* strains. Strains were pre-grown on 100 mM D-glucose and shifted to fresh media containing 50 mM L-arabitol with (A + G) or without (A) 20 mM glycerol and grown further for 6 and 10 h. Activities are expressed in mU/mg mycelial dry weight.

Previous Abf induction studies in *A. nidulans* were limited to enzyme activity measurements, western blotting and inducer-level effects [30,34]. With the availability of the *abfB* gene, we also studied the *abfB* expression at the transcriptional level. As is shown in Fig. 4, the *abfB* transcript accumulation paralleled the total extracellular Abf activity and AbfB protein levels in the culture filtrate. This is in agreement with the results found for the *abf*B-disruption strain in which it was shown that AbfB is the major component of the pNP-A hydrolysing activities in *A. nidulans* (see below). The *abfB* transcription was also analysed when the strains were transferred to media containing 50 mM L-arabitol and 20 mM glycerol as mixed carbon source. Glycerol was added as a non-repressing carbon source to sustain growth of strains carrying the *araA1* mutation, which are unable to utilise L-arabitol (Figs. 3-5). HPLC analysis showed that the concentrations of L-arabitol in the culture filtrates were 30-45 mM after 10 h of growth. Those of glycerol, if added to the cultures, were 4-9 mM (data not shown). Although the

expression levels were lower than in the L-arabitol cultures, the overall pattern of *abfB* expression is very similar. Under the conditions tested, glycerol repressed the L-arabitol-induced *abfB* expression. This repression by glycerol was also found when *A. nidulans* wild-type was cultured directly on 100 mM L-arabinose in combination with 100 mM glycerol [34]. Transcript levels of the *Agaricus bisporus* genes *cel2* and *cel4*, encoding a cellobiohydrolase I and a β-mannanase, respectively, are also moderately repressed when glycerol was added to cellulose-induced cultures [35]. These findings indicate that glycerol can act as a repressing carbon source in different organisms, although it can also be a neutral or inducing carbon source on the expression of different enzymes.

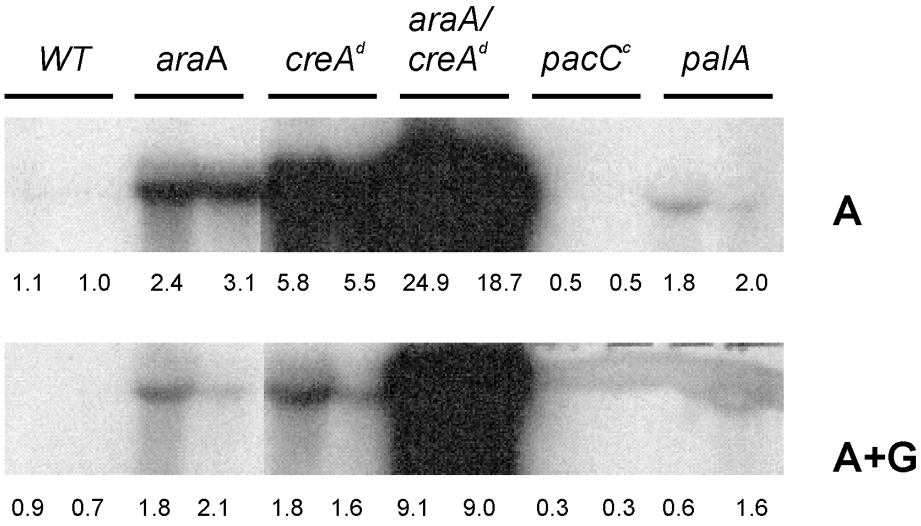

Fig. 4. Northern blot analysis of *abfB* transcription in various *A. nidulans* strains. Strains were pre-grown on 100 mM D-glucose and shifted to fresh media containing 50 mM L-arabitol with (A+G) or without 20 mM glycerol (A) and grown further for 6 and 10 h (first and second lane of each strain, respectively). The relative *abf*B RNA levels, which are expressed in arbitrary units, are also shown. The *abfB* transcript levels were corrected for loading differences and all values were normalised for the sample of WG096 transferred to L-arabitol and grown for 10 h.

As in the case of the *A. nidulans ipnA* [4], *xlnA* and *xlnB* [17] genes, the presence of putative PacC-binding sites suggest possible regulation in response to external pH. Therefore, we have investigated the potential role of PacC by studying AbfB induction in a constitutive mutant (*pacC*^{c}*14*), which mimics growth under alkaline conditions, and in a *pal* mutant (*palA1*), which mimics growth under acidic conditions. The level of pNPase activity was higher in the *palA1* strain than the wild-type, whereas in the *pac*C^{c} strain it

was lower than in the wild-type (Fig. 3). The repression of AbfB induction by the *pacC*^{c}14 mutation is shown in Figs. 4 and 5. Thus it can be concluded that the expression of the *abf*B gene is pH regulated and is higher at acidic pHs.

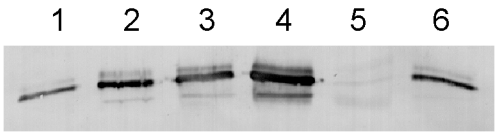

Fig. 5. Western blot analysis of 15 x concentrated culture filtrates using antibodies raised against *A. niger* AbfB. Strains were pre-grown on 100 mM D-glucose and shifted to fresh media containing 50 mM L-arabitol as sole carbon source and grown further for 10 h. Track order; 1: WG096 [wt], 2: G094 [*araA1*], 3: creA^{d}4 [*creA*^{d}*4*], 4: NW186 [*araA1*/*creA*^{d}*4*], 5: pacC^{c} [*pacC*^{c}*14*], 6: palA [*palA1*].

Disruption of the *A. nidulans abfB* gene.

AbfB was found to be the major α-L-arabinofuranosidase activity in *A. nidulans*, since no *abfA* homologue could be detected in *A. nidulans* [21]. We therefore constructed a strain in which the *abfB* gene was disrupted to test this and to explore the possibility that other unknown α-L-arabinofuranosidase functions are present in *A. nidulans*. A linear 5.6 kb *Bam*HI fragment containing the *abfB* gene disrupted by a functional clone of the *A. niger pyrA* gene was derived from the plasmid pIM3005. This fragment was introduced in a genetic background, which displays a high level of Abf activity compared to wild-type, facilitating the possible identification of other minor L-arabinose releasing activities. For this purpose, we first tried to cross in the *pyrG89* allele in a *creA*^{d}*4* background. Despite the distance between the *creA* and *pyrG* alleles (3.6 cM), we only obtained recombinant strains that showed a wild-type phenotype for both alleles. Therefore, we introduced a new *pyrG90* mutation, which was induced by UV-mutagenesis and selected using fluoroorotic acid, in the carbon catabolite derepressed strain carrying the *creA*^{d}*4* allele. The resulting strain, NW187, was used as recipient strain in the disruption experiment. Disruptant NW187::pIM3005-8, designated as NW190, was chosen for further analysis. Strains WG096, NW187 and NW190 were used in transfer cultures as described in Fig. 5. No AbfB protein could be detected in the culture filtrate of the Δ*abfB* strain using *A. niger* AbfB antibodies (Fig. 6a). Furthermore, the pNPase activity was reduced approximately 100-fold to1.2 % of the level seen in control strain NW187 (Fig. 6c). Activities were expressed in mU ml^{-1} since we did not

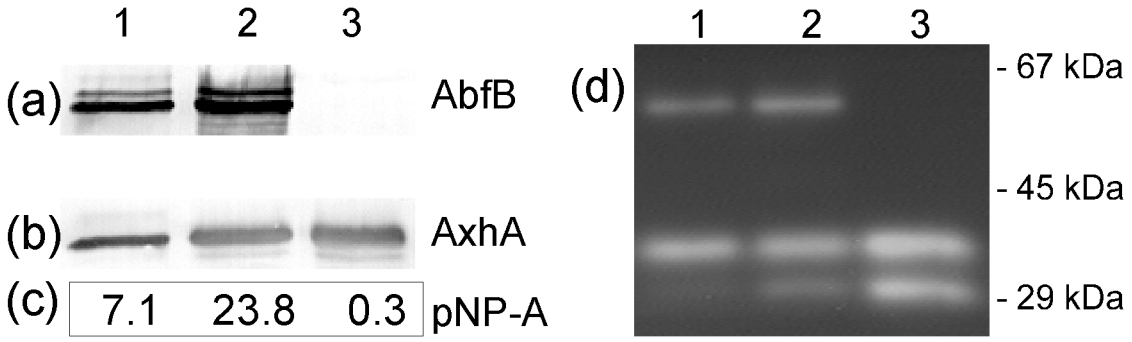

Fig. 6. Western blot analysis of the 15 x concentrated culture filtrates visualised using antibodies raised against *A. niger* AbfB (a) or *A. tubingensis* AxhA (b). (c) pNP-A activities of 15 x concentrated samples are expressed in mU ml^{-1}. (d) SDS-PAGE followed by zymography of the 15 x concentrated culture filtrates visualised using the chromogenic substrate MU-ara. Strains were pre-grown on 100 mM D-glucose and shifted to fresh media containing 50 mM L-arabitol and grown further for 10 h. Track order; 1: WG096 [-], 2: NW187 [*creA*^{d}*4*], 3: NW190 [*creA*^{d}*4*, Δ*abfB*].

determine the actual dry weights of all samples. This reduction in pNPase activity indicates that AbfB is the major component responsible for the observed pNPase values in *A. nidulans*. No AbfB activity could be detected when SDS-PAGE was conducted followed by a renaturation step and active enzyme staining using 4-methylumbelliferryl-α-L-arabinofuranoside (MU-ara). However, two other MU-ara activities having an apparent molecular mass of 30 and 33 kDa, respectively, could be visualised (Fig. 6d). Western blot analysis demonstrated that the 33 kDa band reacted with antibodies raised against *A. tubingensis* arabinoxylan arabinofuranohydrolase A (AxhA)(Fig. 6b). This protein is likely to be the *A. nidulans* equivalent of AxhA from *A. tubingensis*. AxhA was found to release L-arabinose residues from arabinoxylans only [16], and its expression was induced by L-arabitol [10]. The *A. tubingensis* AxhA enzyme has a much higher specific activity towards MU-ara than to pNP-A. The specific activity of *A. tubingensis* AxhA on pNP-A is approximately 1,5 x 10^{-2} U mg^{-1} against 23.5 U mg^{-1} for AbfB [11]. The AxhA equivalent in *A. nidulans* is therefore a significant component of the residual pNPase activity present in the Δ*abfB* strain. Besides AxhA, another MU-ara hydrolysing activity corresponding with a 30 kDa band is present in *A. nidulans*, but it is unknown whether this contributes to the pNP-A hydrolysing activity.

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Chapter 4

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

M.M.C. Gielkens, J. Visser, L.H. de Graaff

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ABSTRACT

The genes encoding arabinoxylan arabinofuranohydrolase, which enzyme 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 overexpression 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 derepressed in *creA*^{d} mutants and carbon catabolite repressed by D-glucose.

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 are involved in this degradation process, which are mainly produced by saprophytic fungi and bacteria. Of the hemicelluloses known, xylan is the most abundant compound. The main chain of this polysaccharide consists of ß-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 [34].

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 to hydrolyse the xylan backbone at non-modified residues. Therefore microorganisms synthesise a number of enzymes capable to remove substituents from the xylan backbone, *e.g.* α-L-arabinofuranosidase B (AbfB)(E.C. 3.2.1.55) [31], acetyl xylan esterase (AxeA)(E.C. 3.1.1.6) [17] and (1,4)-ß-D-arabinoxylan arabinofuranohydrolase

(AxhA) [15]. These enzymes, which were all identified in various *Aspergillus* species, show strong synergistic effects with endoxylanase activity resulting in an enhanced degradation of xylan.

AxhA was first isolated and described by Kormelink *et al.* [15]. This enzyme releases 1,2-α- and 1,3-α-linked arabinofuranosyl groups from arabinoxylans and arabinoxylan-derived oligosaccharides but not from other L-arabinose-containing substrates as arabinans and arabinogalactans [15,16]. This in contrast to AbfB which also shows arabinose-releasing activity towards L-arabinan and arabinogalactan and 1,5-α-linked α-arabinofuranosyl oligosaccharides [31]. From kinetic experiments it was concluded that AxhA is able to release arabinofuranosyl substituents from terminal as well as from non-terminal single substituted xylopyranosyl residues in low molecular weight oligosaccharides, whereas AbfB can only release arabinofuranosyl substituents from terminal single substituted xylopyranosyl residues [16].

In this study we have cloned the *axhA* gene from two related *Aspergilli*. Kusters-van Someren *et al.* [20] 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 its 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 [8]. Differences can also be quantitative, as was reported for the polygalacturonase encoding *pgaII* genes of both strains [3]. 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 [20] and *A. niger* N402 (*cspA1*), 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 analyse *axhA* expression. *Escherichia coli* strains DH5α (Gibco-BRL) LE392, XL1-Blue MRF' were supplied by Stratagene. Phage ExAssist (Stratagene) was used as a helper phage for phagemid excision. Plasmid vectors pBluescript II SK^{-} [29], pUC19 [35], pGEM5, pGEM7 and pGEM-T (Promega) 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* [19].

Aspergillus **cultivation: media and conditions.** All the media used for fungal growth and induction experiments were based on *Aspergillus* minimal medium, which had a composition described by Pontecorvo *et al.* [23]. The final pH was adjusted to 6.0. To the minimal medium carbon sources were added as indicated in the particular experiments. The growth temperature was 30°C in all cases. Mycelium to be used for protoplast formation was pregrown for 18 h on minimal medium with 100 mM D-glucose as carbon source supplemented with 0.2% casamino acids and 0.5% yeast extract. Where necessary the media were supplemented with 10 mM 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) polyvinylidene difluoride membranes [22]. The membrane fragment containing the main band with an apparent molecular mass of 32 kDa, was used for sequence analysis using a gas-phase sequencer equipped with a PTH analyser [1]. In addition, also the sequence of an internal peptide was 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 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) 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 carbon source. Total RNA was isolated by using the guanidium thiocyanate/CsCl protocol [26], 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 [2,26] with the following modification; SDS was omitted from all solutions.

PolyA^+ RNA was pooled and 7 mg was used to synthesise cDNA. 120 ng of cDNA was ligated into 1.2 mg of vector arms of bacteriophage lambda λ Uni-ZAP XR using the ZAP-cDNA synthesis kit (Stratagene) according to the manufacturers instructions. After ligation of the cDNA into Uni-ZAP XR vector arms, the phage DNA was packaged using Packagene extracts (Promega) according to the manufacturers 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.* [7] 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 [26], was amplified using the following sequence: the DNA was heat denatured by incubation for 3 min at 95°C which was followed by 25 cycli 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 [8] was screened by using a 500 bp fragment, generated by PCR and containing *A. niger axhA* cDNA sequences, as probe. The *A. niger* N400 genomic library in λEMBL4 [10] 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 x 10^4 pfu were screened using the following conditions for hybridisation. After prehybridisation of the filters for 2 h in prehybridisation buffer containing 6x SSC (1x SSC: 0.15 M NaCl, 0.015 M Na_3citrate pH 7.6) [26], ^{32}P-labelled probe was added to the prehybridisation solution and hybridised overnight at 65°C. After hybridisation the filters were washed down to 0.1x SSC at 65°C. Other DNA manipulations such as plasmid DNA isolation, λDNA isolation, Southern blot analysis and subcloning, were performed as described by Sambrook *et al.* [26].

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 [27] using the T7 Sequencing Kit (Pharmacia) according to the suppliers 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 [5].

Primer extension mapping to determine transcription initiation sites was performed according to Calzone *et al.* [4] 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 carbon source. RNA was isolated from mycelial powder with TRIZol (Gibco-BRL) according to the manufacturers instructions. PolyA^+ RNA was isolated from 2 mg of total RNA as described above.

Northern analysis. Mycelium to be used transfer experiments was pregrown for 18 h on minimal medium with 100 mM D-fructose as 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 minimal medium and grown for an additional 6-12 h. Total RNA was isolated with TRIZol (Gibco-BRL) according to the manufacturers instructions and 20 mg of total RNA was loaded on formaldehyde agarose gels [26], transferred to Hybond-N membranes (Amersham) and UV-cross linked. Hybridisation and washing of the membranes were carried out essentially as described [26].

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 (8000xg, 10 min) to pellet debris. Proteins secreted into the medium were analysed by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS [21] on a midget gel electrophoresis system (Pharmacia). For the specific detection of AxhA, western analysis was used by incubating nitro-cellulose blots with anti-AxhA antiserum followed by staining with alkaline phosphatase labelled goat anti-rabbit IgG conducted as described by the manufacturer (Bio-Rad).

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 the data reported for AxhA [15]. *The A. tubingensis* AxhA protein was also used to raise antibodies. These antibodies reacted also 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 x 10^4 pfu were immunologically screened for expression of AxhA cDNA. About fifty 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' end of the cDNA. All these clones 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 [10] 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 characterised 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).

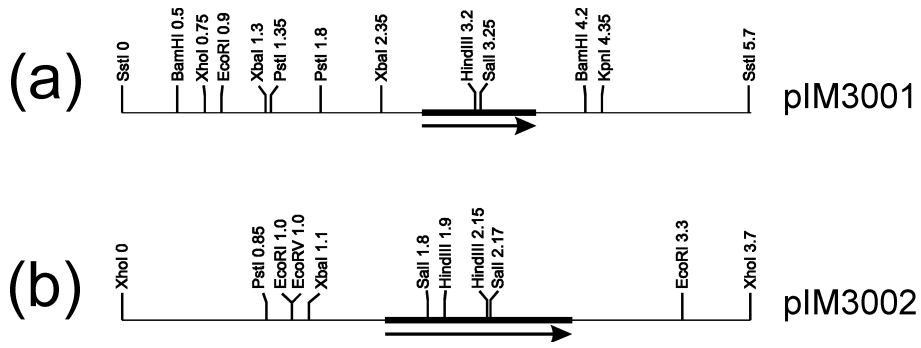
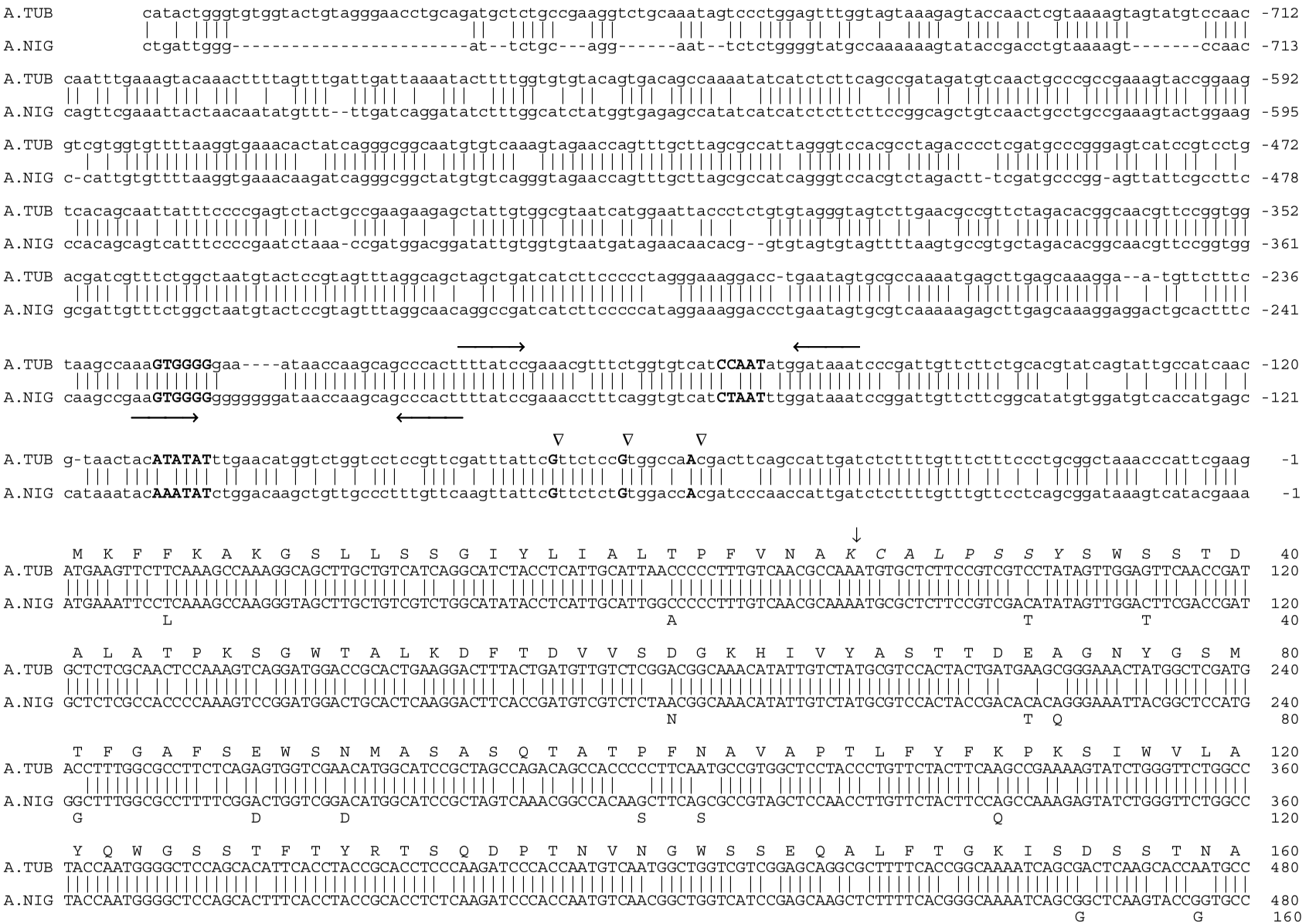


Fig. 1. (a) Restriction map of the 5.5 kb *Sst*I fragment, present on plasmid pIM3001, containing the *A. tubingensis axhA* gene and its flanking regions. (b) Restriction map of the 3.7 kb *Xho*I fragment, present on the plasmid pIM3002, containing the *A. niger axhA* gene and its flanking regions. The position of the *axhA* genes is represented by the black bars, the arrows indicate 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.


A.TUB catactgggtgtggtactgtagggaacctgcagatgctctgccgaaggtctgcaaatagtccctggagtttggtagtaaagagtaccaactcgtaaaagtagtatgtccaac -712
A.NIG ctgattggg----------at--tctgc---agg------aat--tctctggggtatgccaaaaaagtataccgacctgtaaaagt-------ccaac -713
A.TUB caatttgaaagtacaaacttttagtttgattgattaaaatacttttggtgtgtacagtgacagccaaaatatcatctcttcagccgatagatgtcaactgcccgccgaaagtaccggaag -592
A.NIG cagttcgaaattactaacaatatgttt--ttgatcaggatatctttggcatctatggtgagagccatatcatcatctcttcttccggcagctgtcaactgcctgccgaaagtactggaag -595
A.TUB gtcgtggtgttttaaggtgaaacactatcagggcggcaatgtgtcaaagtagaaccagtttgcttagcgccattagggtccacgcctagaccctcgatgcccgggagtcatccgtcctg -472
A.NIG c-cattgtgttttaaggtgaaacaagatcagggcggctatgtgtcagggtagaaccagtttgcttagcgccatcagggtccacgtctagactt-tcgatgcccgg-agttattcgccttc -478
A.TUB tcacagcaattatttccccgagtctactgccgaagaagagctattgtggcgtaatcatggaattaccctctgtgtagggtagtcttgaacgccgttctagacacggcaacgttccggtgg -352
A.NIG ccacagcagtcatttccccgaatctaaa-ccgatggacggatattgtggtgtaatgatagaacaacacg--gtgtagtgtagttttaagtgccgtgctagacacggcaacgttccggtgg -361
A.TUB acgatcgtttctggctaatgtactccgtagtttaggcagctagctgatcatcttccccctagggaaaggacc-tgaatagtgcgccaaaatgagcttgagcaaagga--a-tgttctttc -236
A.NIG gcgattgtttctggctaatgtactccgtagtttaggcaacaggccgatcatcttccccataggaaaggaccctgaatagtgcgtcaaaaagagcttgagcaaaggaggactgcactttc -241
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M K F F K A K G S L L S S G I Y L I A L T P F V N A K C A L P S S Y S W S S T D 40
A.TUB ATGAAGTTCTTCAAAGCCAAAGGCAGCTTGCTGTCATCAGGCATCTACCTCATTGCATTAACCCCCTTTGTCAACGCCAAATGTGCTCTTCCGTCGTCCTATAGTTGGAGTTCAACCGAT 120
A.NIG ATGAAATTCCTCAAAGCCAAGGGTAGCTTGCTGTCGTCTGGCATATACCTCATTGCATTGGCCCCCTTTGTCAACGCAAAATGCGCTCTTCCGTCGACATATAGTTGGACTTCGACCGAT 120
L A T T 40
A L A T P K S G W T A L K D F T D V V S D G K H I V Y A S T T D E A G N Y G S M 80
A.TUB GCTCTCGCAACTCCAAAGTCAGGATGGACCGCACTGAAGGACTTTACTGATGTTGTCTCGGACGGCAAACATATTGTCTATGCGTCCACTACTGATGAAGCGGGAAACTATGGCTCGATG 240
A.NIG GCTCTCGCCACCCCAAAGTCCGGATGGACTGCACTCAAGGACTTCACCGATGTCGTCTCTAACGGCAAACATATTGTCTATGCGTCCACTACCGACACACAGGGAAATTACGGCTCCATG 240
N T Q 80
T F G A F S E W S N M A S A S Q T A T P F N A V A P T L F Y F K P K S I W V L A 120
A.TUB ACCTTTGGCGCCTTCTCAGAGTGGTCGAACATGGCATCCGCTAGCCAGACAGCCACCCCCTTCAATGCCGTGGCTCCTACCCTGTTCTACTTCAAGCCGAAAAGTATCTGGGTTCTGGCC 360
A.NIG GGCTTTGGCGCCTTTTCGGACTGGTCGGACATGGCATCCGCTAGTCAAACGGCCACAAGCTTCAGCGCCGTAGCTCCAACCTTGTTCTACTTCCAGCCAAAGAGTATCTGGGTTCTGGCC 360
G D D S S Q 120
Y Q W G S S T F T Y R T S Q D P T N V N G W S S E Q A L F T G K I S D S S T N A 160
A.TUB TACCAATGGGGCTCCAGCACATTCACCTACCGCACCTCCCAAGATCCCACCAATGTCAATGGCTGGTCGTCGGAGCAGGCGCTTTTCACCGGCAAAATCAGCGACTCAAGCACCAATGCC 480
A.NIG TACCAATGGGGCTCCAGCACTTTCACCTACCGCACCTCTCAAGATCCCACCAATGTCAACGGCTGGTCATCCGAGCAAGCTCTTTTCACGGGCAAAATCAGCGGCTCAAGTACCGGTGCC 480
G G 160

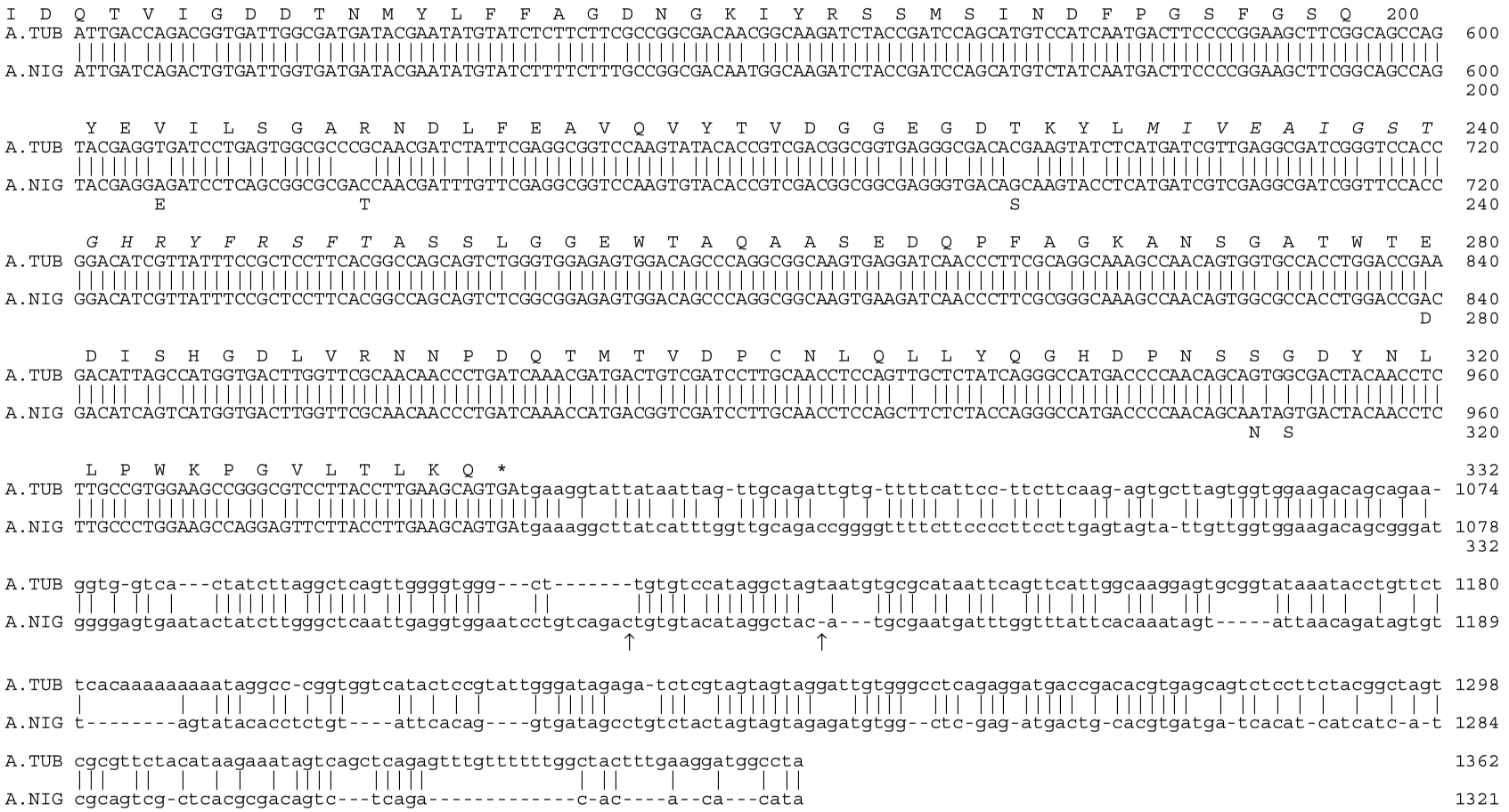

Fig. 2. The nucleotide sequences of the *axhA* genes of *A. tubingensis* (upper sequence) *A. niger* (lower sequence). The deduced amino acid sequence of the *A. tubingensis* AxhA is shown, whereas for the *A. niger* AxhA only those amino acids are shown which are different in the *A. tubingensis* AxhA. The N-terminus of the mature proteins is indicated by "↓". Amino acid residues of *A. tubingensis* AxhA as determined by amino acid sequencing are given in italics. Conserved nucleotides are indicated by "|", whereas alignment improvements by the introductions of insertions are marked by "-". Putative TATA, CAAT and CreA sites are shown bold. Inverted repeats in the upstream non-coding sequence are indicated by arrows. Transcription start points as they were determined are indicated by "∇", while the transcription stops are indicated by "↑". The sequence of the *A. niger* cDNA clone pC61A was found from position -56 to 1169.

An *A. tubingensis* genomic library [8] was screened to isolate the gene encoding AxhA by homologous hybridisation using the 500 bp PCR fragment described above resulting in three hybridising plaques. The inserts of the three phages were characterised 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 *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) 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 nucleotide sequences of both *axhA* genes and their deduced amino acid sequences are shown in Fig. 2. 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 of 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 [9,30] like a TATA and a CAAT box, located respectively 46 bp and 108 bp upstream of the major *tsp*, respectively. 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 of 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 [24].

The coding regions of both genes consisted of a single open reading frame of 996 bp in length, 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 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 [11] for 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.3a, 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 analysed for 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*. Culture filtrates were analysed 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 analysed overexpressed the corresponding gene product (Fig.3). 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.

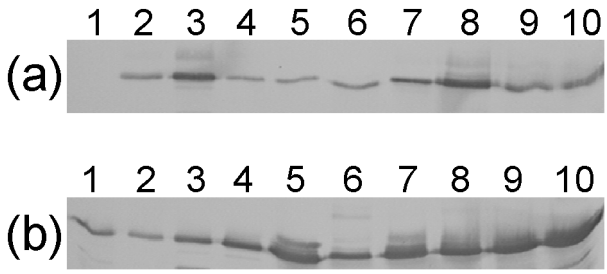

Fig. 3. Western blot analysis of AxhA expression by *A. niger* wild-type N402 and *A. niger* transformants upon growth on minimal medium containing 1.5 % crude wheat arabinoxylan as carbon source. From each strain 20 µl of a culture medium sample was loaded on gel. (a) *A. niger* transformants, containing copies of the *A. tubingensis axhA* gene, were grown for 20 h (lanes 1-5) and 41 h (lanes 6-10). 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. (b) *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 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.

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

The expression of both AxhA and AbfB, encoded by *abfB* [6] 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.4a,c). Northern analysis of *axhA* transcription showed similar results. Transcription of *abfB* was strongly induced by L-arabitol and L-arabinose and only weakly by xylan (Fig.4b,d).

To study the effect of carbon catabolite repression on AxhA expression, two *A. niger* strains carrying *creA*^{d} mutations [33] and the wild-type were pregrown on 100 mM 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 D-glucose and cultured for another 6 h. Both northern and western analysis gave similar results *viz*. AxhA expression was derepressed 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 D-glucose (Fig.5).

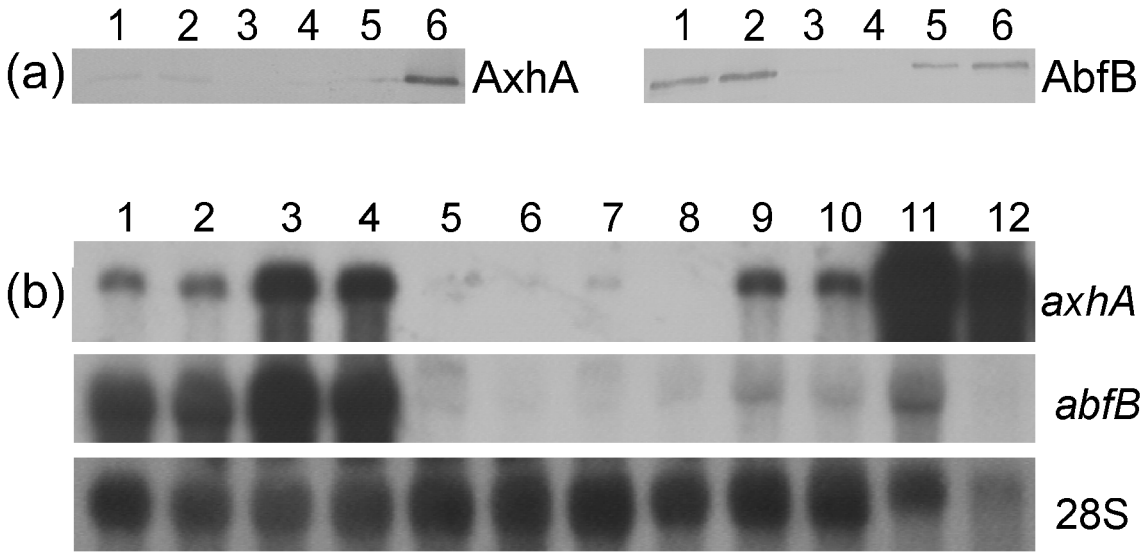

Fig. 4. Expression analysis of the *A. niger axhA* gene. (a) Western analysis of transfer cultures of *A. niger* N402 after 6 h growth. Lane 1: 50 mM L-arabinose; 2: 50 mM L-arabitol; 3: 50 mM D-fructose; 4: 50 mM D-glucose; 5: 50 mM D-xylose; 6: 1% birchwood xylan; visualised with anti-AxhA or anti-AbfB. (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; 7 and 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*, *abfB* [6] or 28S rDNA [28] as loading control.

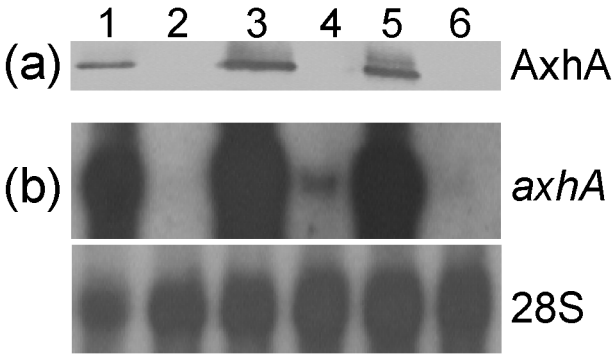

Fig. 5. Expession analysis of *A. niger axhA* in *A. niger* wild-type and strains carrying *creA*^{d}*2* or *creA*^{d}*4* mutations. (a) Western blot analysis and (b) northern analysis of transfer cultures after 6 h growth of N402 (lanes 1 and 2); *cre*A^{d}2 (lanes 3 and 4) and *creA*^{d}*4* (lanes 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).

DISCUSSION

The nucleotide sequences of the genes encoding AxhA in *A. niger* as *A. tubingensis* have been determined and comparison with the EMBL and GenBank databases did not

result in 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 [13]. 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.6).

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

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

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

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

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

AXHNIG   DQPFAGKANSGATWTDDISHGDLVRNNPDQTMTVDPCNLQLLYQGHDPNS  314
AXHTUB   DQPFAGKANSGATWTEDISHGDLVRNNPDQTMTVDPCNLQLLYQGHDPNS  314
XYLC     ANPFAGM---------------MFCFTMASSLKVYTC-----Y-------  571
         ..****.               .   . . ...* .*     *
```

Fig. 6. 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 '.'.

The major *tsp*, 64 bp upstream of the putative start codon, is in both genes preceded 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 have a function in correct initiation of transcription [25]. 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 [18]. 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 promoters of other xylanolytic genes in *Aspergillus*. Downstream of 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 [9]. Although a few AT-rich sequences were found in the 3' non-coding region of both genes, a consensus polyadenylation signal was not present [24].

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*, endoxylanase B (*xynB*), polygalacturonase II (*pgaII*) 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 [32]. Next to comparison of DNA sequence data, the black *Aspergillus* 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).

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)] Ito [12], Kinoshita *et al.* [14]
[c)] Bussink *et al.* [3]
[d)] Visser *et al.* [32]

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.4. Expression of *axhA* is possibly regulated in a similar manner as proposed for the expression of *xlnA* in *A. tubingensis* [8]. Transcription of *axhA* is most likely under direct control of a route-specific transcriptional activator. The northern analysis shown in Fig.5 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 caused by 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* [8].

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Chapter 5

The transcriptional activator XlnR regulates both xylanolytic and endoglucanase expression in *Aspergillus niger*.

N.N.M.E. van Peij, M.M.C. Gielkens, R.P. de Vries,

J. Visser, L.H. de Graaff

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ABSTRACT

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 β-xylosidase, respectively). Also, the transcription of the genes encoding the accessory enzymes involved in xylan degradation, including α-glucuronidase A, acetylxylan esterase A, arabinoxylan 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.

INTRODUCTION

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 β-glucosidases (EC 3.2.1.21). Members of all of these classes are necessary to degrade cellulose, a homopolymer of β-1,4-linked D-glucose. Xylan, however, is a heterogeneous polymer with a backbone consisting of β-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, α-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 co-operative process of xylan breakdown: endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), acetylxylan esterase (EC 3.1.1.72), α-L-arabinofuranosidase (EC 3.2.1.55), arabinoxylan arabinofuranohydrolase, β-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 [1,18,19,23]. It has been shown that xylanase- and cellulase-encoding genes are regulated at the transcriptional level [17,21,30,34]. 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 [17] and arabinanase-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 [20,21] and xylanase-encoding genes [28,30]. 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 [17,19,30,37,48]. Nevertheless, on the basis of biochemical data [1,18,19] and mRNA expression analysis data [21,30], 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 [35]. 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 (*cspA*1), a

short-conidiophore derivative, NW205::130 (*argB*13, *cspA*1, *nicA*1, *pyrA*6, UAS(*xlnA*)-*pyrA*), NXA1-4 (*argB*13, *cspA*1, *nicA*1, *pyrA*6, UAS(*xlnA*)-*pyrA*, *xlnR*1) (strains NW205::130 and NXA1-4 are described more extensively by Peij *et al.*[35], and N902 (*argB*15, *cspA*1, *fwnA*1, *metB*10, *pyrA*5).
Strain N902::230-25.12 (*argB*15, *cspA*1, *fwnA*1, *metB*10), which contains approximately 20 additional copies of *xlnR*, as determined by a phopho-imager analysis of Southern blots, was obtained by cotransformation of *A. niger* N902. The cotransforming plasmids were pIM230 [35] and pGW635 [15], 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 [25].

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^{-1}. 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, 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* [34] was screened for the expression of endoglucanases by using a modified procedure [6,46,47]. Plates contained 20 ml of 2* TY, 0.2% carboxymethylcellulose (CMC) (Sigma), 1.5% agar, and 100 μg 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 5M 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 β-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 μg 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 hybridised at 42°C in a solution containing 50% (vol/vol) formamide, 10% (wt/vol) dextran sulphate, 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 sulphate, and 100 μg of single-stranded herring sperm DNA per ml. Washing was done under homologous hybridisation conditions with a solution containing 30 mM NaCl, 3 mM trisodium citrate, and 0.1% (wt/vol) sodium dodecyl sulphate at 68°C. The ^{32}P-labelled DNA probes used were either cDNA or genomic fragments, as shown in Table 1.

A 1 kb β-glucosidase cDNA fragment of *A. niger*, as determined by sequence analysis, was isolated from a xylan-induced cDNA library [34] by using PCR with degenerate oligonucleotides based on the *Aspergillus kawachii* (EMBL accession no. AB003470) and *Aspergillus aculeatus* (EMBL accession no. P48825) sequences for β-glucosidase and cloned into pGEM-T (Promega).

Table 1. Probes used in northern blot analysis.

Gene	EMBL	Enzyme encoded	Fragment used	Reference
abfB	X74777	α-L-Arabinofuranosidase B	1.7 kb *Eco*RI-*Xho*I	[11]
aguA	Y15405	α-Glucuronidase A	0.8 kb *Eco*RV-*Kpn*I [b]	[44]
axeA	A22880	Acetylxylan esterase A	1.5 kb *Hin*DIII [a, b]	[16]
axhA	Z78011	Arabinoxylan hydrolase A	1.2 kb *Eco*RI-*Xho*I	[13]
bglA	-	β-Glucosidase A	1.0 kb *Nco*I-*Sst*I	This work
eglA	AJ224451	Endoglucanase A	0.9 kb *Xho*I	This work
eglB	AJ224452	Endoglucanase B	1.1 kb *Eco*RI-*Xho*I	This work
faeA	Y09330	Feruloyl esterase A	0.5 kb *Eco*RV-*Xho*I [b]	[43]
xlnB	D38071	Endoxylanase B	0.9 kb *Eco*RI-*Xho*I	[22]
xlnC	-	Endoxylanase C	1.2 kb *Eco*RI-*Xho*I	[14]
xlnD	Z84377	β-Xylosidase D	2.8 kb *Pst*I-*Nsi*I [b]	[34]
18S	X78538	18S rRNA subunit	0.7 kb *Eco*RI	[31]

[a] fragment from the *Aspergillus tubingensis axeA* gene.
[b] genomic fragment instead of cDNA.

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 β-xylosidase encoding genes *xlnB* and *xlnD* [35]. 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 analysed 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 cultures containing 1 % birchwood xylan and to media cultures containing 1 % D-xylose (both birchwood xylan and D-xylose

are known to be carbon sources that induce the xylanolytic system in *A. niger*)[17]. Northern blot analysis of RNA obtained from the wild-type strain showed that xylanolytic, arabinanolytic, 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 [17,38], 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 [35]. 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 effects 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 re-examined 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.

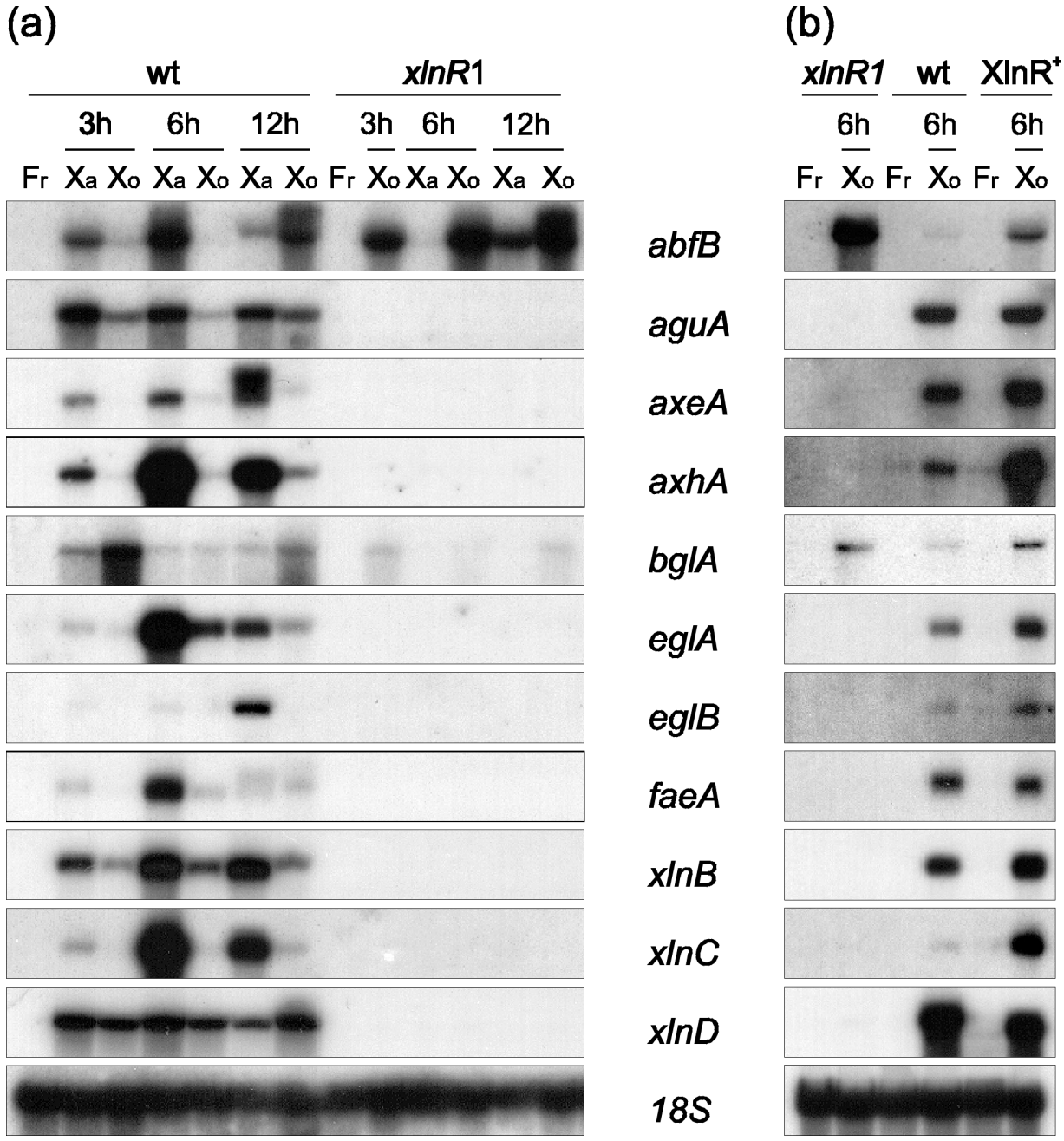

Fig. 1. Northern blot analysis of expression of *A. niger* genes encoding cellulose- and xylan-degrading enzymes. (a) Time course of induction in *A. niger* NW205::130 (wt) and NXA1-4 (*xlnR*1) (a loss-of-function mutant). Both strains were cultured for 18 h in medium containing 3% D-fructose (Fr), and 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 µg of total RNA, which was checked by hybridisation with the 18S rRNA probe. Blots were hybridised 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 (*xlnR*1), and N902-pIM230-25.12 (XlnR$^+$) (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.

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 genes were also expressed. Some genes (for example *aguA* and *faeA*) had equal transcript levels in both the wild-type and *xlnR* multicopy strains, whereas other genes (for example *abfB*, *axhA*, *bglA*, *xlnB* and *xlnC*) had increased transcription levels in the *xlnR* multicopy strain, compared to 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 (endoxylanases B and C and β-xylosidase, respectively). In addition, the *aguA*, *axeA*, *axhA*, and *faeA* genes encoding accessory enzymes (α-glucuronidase A, acetylxylan esterase A, arabinoxylan 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'-GGCTAAA-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 endoxylanase promoters of different *Aspergillus* species [35]. The results presented here shown 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 and that 5'-GGCTAA-3' is a more appropriate consensus sequence, but the seventh nucleotide could play a role in XlnR binding.

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

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

[a] position relative to the ATG translation start codon. (R) indicates the opposite orientation of the putative XlnR binding site

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 [35].

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 [26].

It has been proposed that repression by CreA of the xylanolytic genes [17,35] is analogous to the double lock mechanism described for the ethanol regulon in *A. nidulans*

[9,24]. In this model CreA represses both the positive and autoregulated *trans*-acting gene *alcR* and structural genes such as *alcA* and *aldA* [10,23,26,29]. Some CreA binding sites in the *alcA* and *alcR* upstream region are close to or overlap the AlcR targets [10,24]. 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 *trans*-acting factors AmdR, FacB, AmdA, AmdX, AreA, and CreA [8,27,32,33]. The overlap of AmdX binding sites with CreA and AmdA binding sites suggests that there is competition for binding sites by multiple factors [32]. The repressor protein CreA has been shown to also have a function in regulation of xylanolytic gene expression in *A. niger* [13,17]. 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 [34]. 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 β-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]. The *abfB* gene expression is under coordinate control with expression of the arabinofuranosidase A-encoding *abfA* gene and the endoarabinase encoding *abnA* gene [12]. 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, but the *abfB* gene does not contain the XlnR binding site. The increase in the level 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 that occur in nature. The fact that expression of the xylanolytic enzymes and expression of some cellulolytic enzymes are co-ordinately 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, β-xylosidase XlnD and β-glucosidase BglA, both of which are members of the family 3 glycosyl hydrolases [34]. Here we provide evidence that there is co-ordination in the regulation of xylanases and some cellulases.

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Chapter 6

Two cellobiohydrolase-encoding genes from *Aspergillus niger* require D-xylose and the xylanolytic transcriptional activator XlnR for their expression.

M.M.C. Gielkens, E. Dekkers, J. Visser, L.H. de Graaff

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ABSTRACT

Two cellobiohydrolase encoding genes, *cbhA* and *cbhB*, have been isolated from the filamentous fungus *Aspergillus niger*. The deduced amino acid sequence shows that CbhB has a modular structure consisting of a fungal-type cellulose-binding domain (CBD) and a catalytic domain separated by a Pro/Ser/Thr-rich linker peptide. CbhA consists only of a catalytic domain and lacks a CBD and linker peptide. Both proteins are homologous to fungal cellobiohydrolases in family 7 of the glycosyl hydrolases. Northern blot analysis shows that the transcription of the *cbhA* and *cbhB* genes is induced by D-xylose and not by sophorose, and furthermore required the xylanolytic transcriptional activator XlnR.

INTRODUCTION

Cellulose or β-1,4-glucan is the most abundant polysaccharide in nature and is closely associated in plant cells walls with the hemicellulose xylan [4]. Filamentous fungi, in particular *Aspergillus* and *Trichoderma* species, are well-known and efficient producers of plant cell wall degrading enzymes. The cellulose degrading system of these organisms consists of three classes of enzymes [3]: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). Members of all these classes are necessary to degrade cellulose.

The most studied fungal cellulolytic system is that of *Trichoderma reesei*. Of the proteins secreted by *T. reesei*, more than 60% is cellobiohydrolase I (CBHI), which is the major component of the cellulase system and plays a central role in the degradation of crystalline cellulose [36]. More recently, the genes for CbhI from *Trichoderma viride, Agaricus bisporus, Penicillium janthinellum, Phanerochaete chrysosporium, Humicola grisea, Neurospora crassa* and *Aspergillus aculeatus* have been characterised [1,5,6,21,35,37]. All but one of these CbhI proteins consist of a catalytic domain and a cellulose binding domain (CBD) linked by a Pro/Ser/Thr-rich linker peptide.

The expression of cellulose degrading enzymes by *Aspergillus* and *Trichoderma* species has been studied extensively [2,15,16,22]. It has been shown that cellulase encoding genes are regulated at the transcriptional level [17,26,29]. In the presence of D-

glucose, the genes are not expressed and the carbon catabolite repressor protein Cre1 in *T. reesei* causes transcriptional repression of some (hemi-)cellulase encoding genes [17,18]. However, less is known about the mechanism by which the transcription of cellulase encoding genes is induced. Recently, it was demonstrated that the *Aspergillus niger* xylanolytic transcriptional activator XlnR also directs the transcription of two endoglucanase encoding genes, *eglA* and *eglB* [28]. Here, we describe the cloning and characterisation of two cellobiohydrolase encoding genes (*cbhA* and *cbhB*) in *A. niger* and demonstrate that XlnR is also involved in the regulation of transcription of these Cbh encoding genes.

MATERIALS AND METHODS

Strains and culture conditions. All *A. niger* strains used were derived from the wild type strain N400 (CBS 120.49). Strains used were N402 (*cspA1*), NW188 (*prtF28*, *goxC1*, *cspA1*, *leuA1*, *pyrA6*), NW188::pIM3012-115 (which contains the CbhA expression construct), NW188::pIM3011-34 (which contains the CbhB expression construct), NW197 (*argB15*, *fwnA6*, *nicA1*, *cspA1*, Δ*xlnR-argB*$^{+}$), N902::pIM230-3.9 (*argB15*, *fwnA1*, *metB10*, *cspA1*, *pyrA5*, *xlnR*$^{+}$-*pyrA*$^{+}$)[10 *xlnR* copies], N902::pIM230::pIM101-6 (20 copies of the *A. tubingensis xlnA* gene(10)), N902::pIM230::pIM101-10 (6 *xlnA* copies), and N902::pIM230::pIM101-12 (2 *xlnA* copies). Copy numbers of the various genes have been determined by the quantification of Southern blots by PhosphorImager analysis (Molecular Dynamics). Signals were corrected for the amount of DNA loaded in each lane by using the signal of the endogenous *abfB* gene.

All media had a pH of 6 and were based on *Aspergillus* minimal medium [31] with the carbon sources as indicated in the figures. Spores were inoculated at 10^6 ml^{-1}. In transfer experiments the pre-cultures with D-fructose were supplemented with 0.2% (w/v) casamino acids and 0.2% (w/v) yeast extract. After 18 h of growth, mycelia were recovered by filtration and washed with minimal medium without carbon source. These mycelia were transferred to minimal medium containing thecarbon sources as indicated in the figures.

Amino acid sequence determination. *A. niger* was grown for 96 h at 30°C in minimal medium supplemented with 1.5 % (w/v) wheat arabinoxylan. The culture filtrate was collected after filtration, diluted three times with water and adjusted to a pH of 6.0. DEAE-Sephadex A-50, equilibrated in 50 mM sodium acetate buffer (pH 5.0), was added to the culture filtrate. After 30 to60 min of stirring at 4°C, the DEAE-Sephadex was collected by filtration and transferred to a column. Protein from this column was first eluted with 50 mM sodium acetate buffer (pH 5.0), and then with 50 mM sodium acetate buffer (pH 5.0) plus 0.5 M NaCl. Pooled fractions were applied on a DEAE-Sepharose Fast Flow column, and protein was eluted from this column with a linear gradient of 0.5 M NaCl in 20 mM piperazine-HCl buffer pH 5.0. The next fractionation step was conducted with a Sephacryl S-300 column, from which protein was eluted with 20 mM piperazine-HCl (pH 5.0)-0.1 M NaCl. Subsequently, a Superdex 75 column (Hiload 6/60)(Amersham Pharmacia Biotech) and protein was

loaded and eluted with 20 mM piperazine-HCl (pH 5.0)-0.1 M NaCl. The final purification was done on a Mono S cation exchange column (HR 5/5,Amersham Pharmacia Biotech). Protein was eluted with a linear gradient of 1 M NaCl in 10 mM sodium acetate buffer (pH 3.5). These fractions were enriched in cellobiohydrolase activity. Trypsic digests were made by EUROSEQUENCE, and peptides were separated to determine their amino acid sequences. Edman degradation was performed with an automated sequenator (Model 477A, Perkin-Elmer Applied Biosystems) coupled to a high-performance liquid chromatograph (HPLC)(Model 120A, Perkin-Elmer Applied Biosystems) for analysis of the phenylthiodantoin amino acids.

PCR. The region encoding the mature protein of the *A. bisporus cel2* gene [38] was amplified by PCR with the oligonucleotides CEL2MAT (5'-GTCGGTACCAACATGGCCG-3') and CEL2STOP (5'-ACTCAGAAACATTGGCTATAG-3') and a full size cDNA clone of *cel2* as the template. The amino acid sequences of the internal peptide fragments of the purified *A. niger* cellobiohydrolase were used to derive the oligonucleotide mixtures AD2 (5'-GAYGAYAGYAAYTAYGARCTNTTYAA-3') and AD6 (5'-GTRAANGGRCTRTTNGTRTC-3'). These oligonucleotide mixtures were used in a PCR with an excised phagemid library, derived from a xylan-induced cDNA library of *A. niger* [8], as a template. The DNA was heat-denatured by incubation for 5 min at 94°C followed by 24 cycles of 1 min at 94°C, 1.5 min at the annealing temperature, and 1.5 min at 72°C. The annealing started at 48°C and was lowered in each cycle by increments of 0.3 to 40°C. Then, ten additional cycles of 1 min at 94°C, 1.5 min at 40°C and 1.5 min at 72°C were conducted. The reaction was terminated after a final 5 min incubation at 72°C.

Isolation, cloning and characterisation of the *A. niger cbhA* and *cbhB* genes. Plaque hybridisation with Hybond-N filters (Amersham Pharmacia Biotech) was performed as described by Sambrook *et al.* [33]. For the isolation of a cDNA clone of *A. niger cbhA*, a xylan-induced cDNA library of *A. niger* [8] was screened with a 1.5 kb PCR fragment containing *Agaricus bisporus cel2* sequences as a probe. Hybridisation was performed overnight at 56°C. The filters were washed with SSC and SDS (final concentrations, 0.5x and 0.5%, respectively [1*SSC contains 0.15 M NaCl and 0.015 M sodium citrate]. All other hybridisations were performed overnight at 65°C, and filters were washed until concentrations of 0.2*SSC and 0.1% SDS were reached. The *A. niger cbhA* and *cbhB* genes were isolated after screening of an *A. niger* N400 genomic library in λEMBL4 [12]. Standard methods were used for other DNA manipulations, such as Southern blot analysis, subcloning, DNA digestions, and λ phage and plasmid isolations [33]. Sequence reactions were performed with a Thermo-Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham Pharmacia Biotech) with universal sequencing primers and a Thermo-Sequenase dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) with gene-specific oligonucleotides. The sequencing reactions were analysed on an ALF*express* sequencer (Amersham Pharmacia Biotech). Nucleotide sequences were determined for both strands, while the coding regions were also determined by sequencing of the cDNA. Sequence analysis was performed with the WinStar software package (DNASTAR). Database searches were performed using the National Center for Biotechnology Information BLAST software.

Expression vectors for the *A. niger cbhA* and *cbhB* genes. The *cbhA* gene was fused to the promoter of the *A. niger pkiA* gene [9] at its start codon with a 3.5 kb *Nsi*I genomic fragment, resulting in pIM3012. This fragment includes the coding region and 3' non-coding flanking region of the *cbhA* gene. A cDNA clone of *cbhB* was modified using PCR. A *Nsi*I restriction site was introduced at the ATG start codon and a *Bam*HI restriction was introduced directly downstream of the stop codon. The *cbhB* gene was fused to the promoter of the *A. niger pkiA* gene at its start codon. The terminator of the *Aspergillus*

nidulans trpC gene was ligated downstream of the *cbhB* stop codon, resulting in pIM3011. Transformation was performed as described previously by Kusters-van Someren *et al.* [24].

Northern blot analysis. Total RNA was isolated from powdered mycelia with TRIzol Reagent (Life Technologies), according to the supplier's instructions. Poly(A)$^+$ mRNA was isolated with the PolyATract system IV (Promega), according to the manufacturer's instructions. For northern blot analysis, 10 μg of total RNA or 2 μg of poly(A)$^+$ mRNA was glyoxylated and separated on a 1.5% (w/v) agarose gel [33]. After capillary blotting to Hybond-N membrane (Amersham Pharmacia Biotech), the transfer and amount of RNA were checked by staining the rRNA on the Hybond filter in a 0.2% (w/v) methylene blue solution. Filters were hybridised at 42°C in a solution of 50% (v/v) formamide, 10% (w/v) dextran sulphate, 6*SSC, 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) bovine serum albumin, 0.1% (w/v) SDS and 100 μg single stranded herring sperm DNA ml^{-1}. Washes were performed under homologous hybridisation conditions to 0.2*SSC and 0.1% (w/v) SDS at 65°C. The ^{32}P-labelled DNA probes used were the cDNA fragments listed in Table 1.

Nucleotide sequence accession numbers. *cbhA* and *cbhB* sequences have been deposited in the GenBank and EMBL sequence databases under accession no. AF156268 and AF156269, respectively.

Table 1. Probes used in northern blot analysis

Gene	EMBL accession no.	Enzyme encoded	Fragment used	Reference
ActA	M22869	Actin	1.6-kb *Nco*I-*Kpn*I[a)]	7
BglA		β-Glucosidase A	1.0-kb *Nco*I-*Sst*I	29
CbhA	AF156268	Cellobiohydrolase A	1.7-kb *Eco*RI-*Xho*I	This study
CbhB	AF156269	Cellobiohydrolase B	1.8-kb *Eco*RI-*Xho*I	This study
EglA	AJ224451	Endoglucanase A	0.9-kb *Xho*I	29
eglB	AJ224452	Endoglucanase B	1.1-kb *Eco*RI-*Xho*I	29
xlnB	D38071	Endoxylanase B	0.9-kb *Eco*RI-*Xho*I	19
18S	X78538	18S rRNA subunit	0.7-kb *Eco*RI	27

a) Genomic fragment from the *Aspergillus nidulans actA* gene.

RESULTS

Cloning and analysis of the primary stucture of the *A. niger cbhA* gene.

Fractions enriched in cellobiohydrolase activities were obtained after fractionation of culture filtrate of *A. niger* grown on arabinoxylan. The conditions were the same as those used to purify endoglucanases A and B and clone their corresponding genes [29]. The protein was enzymatically hydrolysed with trypsin, and from two of the internal

peptides obtained, we determined the N-terminal amino acid sequences, specifically, L Y L M S D D S N Y E L F K (S1) (14 residues) and L G N T D F Y G P G L T V D T N S P F T V V T Q (S2) (24 residues). Both sequences showed high identity to a cDNA clone of *cel2* from *Agaricus bisporus* [38], which encodes a cellobiohydrolase. Screening of a xylan-induced cDNA library of *A. niger* [8] with this PCR fragment carrying this gene resulted in the isolation of a full-length cDNA clone, designated CbhA-C9. This cDNA clone was subsequently used as a probe to screen an *A. niger* N400 genomic library [12]. A 9 kb *Eco*RI fragment containing the *cbhA* gene was cloned resulting in pIM3010.

The sequence determined for the *cbhA* gene was 3498 bp long and contained 1130 bp of the 5' non-coding region and 857 bp of the 3' non-coding region. In the promoter region, one putative binding site for the CreA protein [23], involved in carbon catabolite repression, was found at position -934. Also, one putative XlnR binding site [28] was found 731 bp upstream of the ATG translation start codon.

The structural part of the *cbhA* gene is interrupted by three introns. All three introns fit the features that are generally found for introns in genes from filamentous fungi [11]. These introns and their positions were confirmed by sequencing the cDNA clone CbhA-C9. By removing the intron sequences, an open reading frame consisting of 451 amino acids which had a putative pre-sequence of 17 amino acids was found. The pre-sequence has all the characteristics of a typical signal peptide [13]. However, the N-terminal amino acid sequences as determined for the internal tryptic fragments were not found in the derived amino acid sequence. Thus, *cbhA* does not encode the cellobiohydrolase activity found in the enzyme fraction. We noticed in addition that CbhA consists only of a catalytic domain and lacks both the cellulose binding domain and the linker peptide, which generally links both domains in fungal cellobiohydrolases.

Cloning and analysis of the primary structure of the *A. niger cbhB* gene.

Degenerate oligonucleotide primers were designed to isolate the gene corresponding to the trypsic peptides. These primers were used on DNA from the template xylan-induced cDNA library [8] in a touch-down PCR protocol. A 500 bp PCR fragment, showing high homology with fungal cellobiohydrolase genes, was used to isolate the full-length cDNA clone CbhB-C1. This clone was used as a probe to isolate the *cbhB* gene, which was present on a 5.5 *Kpn*I fragment and subsequently cloned, resulting in pIM3013.

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ANCBHA  M-------HQRALLFSALLTAVR-AQQAGTLTEEVHPSLTWQKCTSEGSCTEQSGSVVIDSNWRWTHSVNDSTNCYTGNTWDATLCPDDETCAANCALDGADYESTYGV  101
ANCBHB  M-SS--FQIYRAALLLS-ILATAN-AQQVGTYTTETHPSLTWQTCTSDGSCTTNDGEVVIDANWRWVHSTSSATNCYTGNEWDTSICTDDVTCAANCALDGATYEATYGV  105
AACBHI  MVDS--FSIYKTALLLS-MLATSN-AQQVGTYTAETHPSLTWQTCSGSGSCTTTSGSVVIDANWRWVHEVGGYTNCYSGNTWDSSICSTDTTCASECALEGATYESTYGV  106
PJCBHI  MKGSISYQIYKGALLLSALLNSVS-AQQVGTLTAETHPALTWSKCTA-GXCSQVSGSVVIDANWPXVHSTSGSTNCYTGNTWDATLCPDDVTCAANCAVDGAR-RQHLRV  108
TRCBHI  M-------YRKLAVISAFLATAR-AQSACTLQSETHPPLTWQKCSSGGTCTQQTGSVVIDANWRWTHATNSSTNCYDGNTWSSTLCPDNETCAKNCCLDGAAYASTYGV  101
ABCEL2  M-------FPRSILLALSLTAVALGQQVGTNMAENHPSLTWQRCTSSG-CQNVNGKVTLDANWRWTHRINDFTNCYTGNEWDTSICPDGVTCAENCALDGADYAGTYGV  101
PCCBHI  MVD-IQIATF---LLLGVVGVA---AQQVGTYIPENHPLLATQSCTASGGCTTSSSKIVLDANRRWIHSTLGTTSCLTANGWDPTLCPDGITCANYCALDGVSYSSTYGI  103
        *              ....  . .    .*...*    * ** *..   *.. * *.    . ...*.*... *      *.*. .* *.   .*..   *** .*...*. .  ....

ANCBHA  TTDGDSLTLKFVTGS---NVGSRLYLMDTSDEGYQTFNLLDAEFTFDVDVSNLPCGLNGALYFTAMDADGGVSKYPANKAGAKYGTGYCD-SQCPRDLKFIDGQANVDGW  207
ANCBHB  TTSGSELRLNFVTQGSSKNIGSRLYLMSD-DSNYELFKLLGQEFTFDVDVSNLPCGLNGALYFVAMDADGGTSEYSGNKAGAKYGTGYCD-SQCPRDLKFINGEANCDGW  213
AACBHI  TTSGSSLRLNFVTTASQKNIGSRLYLLAD-DSTYETFKLFNREFTFDVDVSNLPCGLNGALYFVSMDADGGVSRFPTNKAGAKYGTGYCD-SQCPRDLKFIDGQANIEGW  214
PJCBHI  TTSGNSLRINFVTTASQKNIGSRLYLLEN-DTTYQKFNLLNQEFTFDVDVSNLPCGLNGALYFVDMDADGGMAKYPTNKAGAKYGTGYCD-SQCPRDLKFINGQANVDGW  215
TRCBHI  TTSGNSLSIGFVTQSAQKNVGARLYLMAS-DTTYQEFTLLGNEFSFDVDVSQLPCGLNGALYFVSMDADGGVSKYPTNTAGAKYGTGYCD-SQCPRDLKFINGQANVEGW  209
ABCEL2  TSSGTALTLKFVTESQQKNIGSRLYLMAD-DSNYEIFNLLNKEFTFDVDVSKLPCGLNGALYFSEMAADGGMS--STNTAGAKYGTGYCD-SQCPRDIKFIDGEANSEGW  207
PCCBHI  TTSGSALRLQFVTGT---NIGSRVFLMAD-DTHYRTFQLLNQELAFDVDVSKLPCGLNGALYFVAMDADGGKSKYPG-RAGAKYGTGYCDASQCPRDVQFINGQANVQGW  208
        *..*  * . ***    .*.*.*..*.   *   *  * *.  *..****** ***********. *.**** . .. . *********** ******..** *.**  **

ANCBHA  EPSSNNDNTGIGNHGSCCPEMDIWEANKISTALTPHPCDSSEQTMCEGNDCGGTYSD--DRYGGTCDPDGCDFNPYRMGNDSFYGPGK--TIDTGSKMTVVTQFITDG--  311
ANCBHB  EPSSNNVNTGVGDHGSCCAEMDVWEANSISNAFTAHPCDSVSQTMCDGDSCGGTYSASGDRYSGTCDPDGCDYNPYRLGNTDFYGPGL--TVDTNSPFTVVTQFITDDGT  321
AACBHI  EPSSTDVNAGTGNHGSCCPEMDIWEANSISSAFTAHPCDSVQQTMCTGDTCGGTYSDTTDRYSGTCDPDGCDFNPYRFGNTNFYGPGK--TVDNSKPFTVVTQFITHDGT  322
PJCBHI  TPSKNDVNSGIGNHGSCCAEMDIWEANSISNAVTPHPCDTPSQTMCTGQRCGGTYS--TDRYGGTCDPDGCDFNPYRMGVTNFYGPGE--TIDTKSPFTVVTQFLTNDGT  321
TRCBHI  EPSSNNANTGIGGHGSCCSEMDIWEANSISEALTPHPCTTVGQEICEGDGCGGTYSD--NRYGGTCDPDGCDWNPYRLGNTSFYGPGSSFTLDTTKKLTVVTQFETSGA-  316
ABCEL2  EGSPNDVNAGTGNFGACCGEMDIWEANSISSAYTPHPCREPGLQRCEGNTCS-----VNDRYATECDPDGCDFNSFRMGDKSFYGPGM--TVDTNQPITVVTQFITDNGS  310
PCCBHI  NATSAT--TGTGSYGSCCTELDIWEANSNAAALTPHTCTNNAQTRCSGSNCTSN--------TGFCDADGCDFNSFRLGNTTFLGAGMS--VDTTKTFTVVTQFITSDNT  306
        .....   . *.* .*.** *.*.****... * *.*.*      .   * *   *.....    ... ..**.****.*..* *.. *.*.*    ..*.    .******.*

ANCBHA  -SGSLSEIKRYYVQNGNVIANADSNISGV-TGNSITTDFCTAQKKAFGDEDIFAEHNGLAGISDAMSS-MVLILSLWDDYYASMEWLDSDYPENATATDPGVARGTCDSE  418
ANCBHB  SSGTLTEIKRLYVQNGEVIANGASTYSSV-NGSSITSAFCESEKTLFGDENVFDKHGGLEGMGEAMAKGMVLVLSLWDDYAADMLWLDSDYPVNSSASTPGVARGTCSTD  430
AACBHI  DTGTLTEIRRLYVQNGVVIGNGPSTYTAA-SGNSITESFCKAEKTLFGDTNVFETHGGLSAMGDALGDGMVLVLSLWDDHAADMLWLDSDYPTTSCASSPGVARGTCPTT  431
PJCBHI  STGTLSEIKRFYVQGGKVIGNPQSTIVGV-SGNSITDSWCNAQKSAFGDTNEFSKHGGMAGMGAGLADGMVLVMSLWDDHASDMLWLDSTYPTNATSTTPGAKRGTCDIS  430
TRCBHI  -------INRYYVQNGVTFQQPNAELGSY-SGNELNDDYCTAEEAEFGGSS-FSDKGGLTQFKKATSGGMVLVMSLWDDYYANMLWLDSTYPTNETSSTPGAVRGSCSTS  417
ABCEL2  DNGNLQEIRRIYVQNGQVIQNSNVNIPGIDSGNSISAEFCDQAKEAFGDERSFQDRGGLSGMGSALDRGMVLVLSIWDDHAVNMLWLDSDYPLDASPSQPGISRGTCSRD  420
PCCBHI  STGNLTEIRRFYVQNGNVIPNSVVNVTGIGAVNSITDPFCSQQKKAFIETNYFAQHGGLAQLGQALRTGMVLAFSISDDPANHMLWLDSNFPPSANPAVPGVARGMCSIT  416
.          . . .*.* ***.* .. .          .....  .*   .   *..    *   ..*.    . .   .***. *..** .   *.**** .*       . **   **.*

ANCBHA  SGVPATVEGAHPDSSVTFSNIKFGPINSTFSASA---------------------------------------------------------------------------  452
ANCBHB  SGVPATVEAESPNAYVTYSNIKFGPIGSTYSSGSSSGSGSSSSSSSTTTKATSTTLKTTSTTS----SGSSSTSAAQAYGQCGGQGWTGPTTCVSGYTCTYENAYYSQCL  536
AACBHI  TGNATYVEANYPNSYVTYSNIKFGTLNSTYSGTSSGGSSSSSTTLTTKASTSTTSSKTTTTTSKTSTTSSSSTNVAQLYGQCGGQGWTGPTTCASG-TCTKQNDYYSQCL  540
PJCBHI  R-RPNTVESTYPNAYVIYSNIKTGPLNSTFTGGTT--SSSSTTTTTSKSTSTSSSSKTTTTVTTTTTSSGSSGTGARDWAQCGGNGWTGPTTCVSPYTCTKQNDWYSQCL  537
TRCBHI  SGVPAQVESQSPNAKVTFSNIKFGPIGST---GNPSGGNPPGGNRGT-----------TTTRRPATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL  513
ABCEL2  SGKPEDVEANAGGVQVVYSNIKFGDINSTFNNNGGGGGNPSP-----------------------TTTRPNSPAQTMWGQCGGQGWTGPTACQSPSTCHVINDFYSQCF  506
PCCBHI  SGNPADVGILNPSPYVSFLNIKFGSIGTTFRPA----------------------------------------------------------------------------  449
        .. .. *.        *  .***.*    .*                                                  .... .  ... . .  ..  .  ....
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Fig.1. (See former page). Amino acid sequence comparison of *A. niger* CbhA (AnCbhA), *A. niger* CbhB (AnCbhB), *P. chrysosporium* CbhI-1 (PcCbhI)[6], *A. aculeatus* CbhI (AaCbhI)[34], *A. bisporus* Cel2 (AbCel2)[37], *P. janthinellum* CbhI (PjCbhI)[21] and *T. reesei* CbhI (TrCbhI)[33]. Aligment was performed with the CLUSTAL V program Identical amino acids (*) and 5 out of 7 amino acids identical (.) are shown.

The sequence determined for the *cbhB* gene was 2622 bp long and contained 607 bp of the 5' non-coding region and 407 bp of the 3' non-coding region. In the promoter region, two putative CreA binding sites [23] were found at positions -444 and -502. One putative XlnR binding site [28] was found at position -157.

The structural part of the *cbhB* gene did not contain introns. The absence of introns was confirmed by sequencing the cDNA clone ChbB-C1. The derived polypeptide sequence consists of 536 amino acids and contains a pre-sequence of 21 amino acids, which complies to the (-3,-1) rule as proposed by von Heine [13]. Both amino acid sequences determined for the two trypsic fragments were found in the derived amino acid sequence. Thus, we can conclude that *cbhB* encodes the cellobiohydrolase activity found in the purified enzyme fraction.

Alignment of the amino acid sequences of CbhA and CbhB with other fungal cellobiohydrolases.

The deduced amino acid sequences of CbhA and CbhB were aligned with the deduced amino acid sequences of other fungal cellobiohydrolases from family 7 of the glycosyl hydrolases [14](Fig. 1). CbhA showed the highest similarity with CbhB (65.3%) and CbhI from *P. janthinellum* [20](62.9%). CbhB showed the highest similarity with CbhI from *A. aculeatus* [35](72.6%).

Functionality of the *cbhA* and *cbhB* genes.

Both genes were fused at their ATG translation start codons to the constitutive promoter of the *A. niger pkiA* gene [9]. This enables expression of these genes under conditions where normally no induction of cellulases occurs. However, various but low levels of endoglucanase activity were still present under these conditions of cultivation, as was concluded after we conducted isoelectric focussing followed by activity staining.

This endoglucanase activity was confirmed by incubation of carboxymethyl cellulose (CMC) with culture filtrate of the parental strain followed by HPLC analysis (data not shown). After introduction of these expression constructs in *A. niger*, transformants were screened for expression of cellobiohydrolase activity with the chromogenic substrate 4-methylumbelliferryl-β-cellobiose (MU-C). Hydrolysis products of this substrate are fluorescent when they are excited by UV light. Transformants which gave the largest halo were selected for submerged cultivation. Southern blot analysis confirmed the integration into the genome of additional copies of the expression constructs carrying the respective *cbh* gene (data not shown). Table 2 shows the cellobiohydrolase activities

Table 2. Cellobiohydrolase activities determined in culture filtrates of recombinant *A. niger* strains NW188::pIM3012-115 and NW188::pIM3011-34[a]. which produce CbhA and CbhB respectively.

Strain	Cellobiohydrolase activity[b] After cultivation lasting:	
	24 h	40 h
NW188	2.3	5.2
NW188::pIM3012-115 (CbhA)	171	201
NW188::pIM3011-34 (CbhB)	220	252

[a] NW188::pIM3012-115 and NW188::pIM3011-34 produce CbhA and CbhB, respectively. The parent strain NW188 was used as a control.
[b] Activities are expressed in microunits of mycelia (dry weight) per milligram.

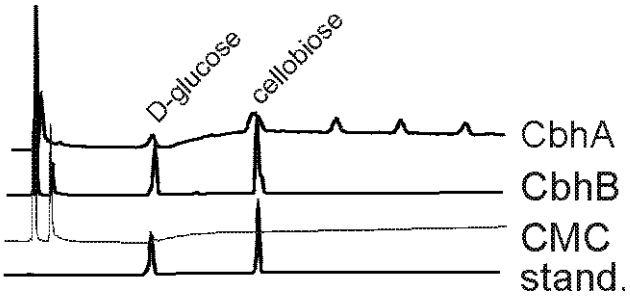

Fig. 2. Saccharification of cellulose by CbhA and CbhB. 1% (w/v) cellulose was digested with enzyme solutions enriched in CbhA or CbhB overnight at 30°C. 50 µl of twofold-diluted heat-inactivated (5 min, 100°C) samples was analysed by high-performance anion-exchange chromatography on a Dionex system with a Carbopac PA-100 column and pulsed amperometrical detection using a gradient of 0.05 M-0.90 M NaOH suitable for glucose oligosaccharide separation. Standards used are D-glucose and cellobiose.

determined from the culture filtrates after cultivation of these transformants on 5% D-glucose. The transformants clearly demonstrated elevated cellobiohydrolase activity, indicating that both genes encode functional cellobiohydrolases. HPLC analysis (Fig. 2) revealed that the enzyme preparations enriched in either CbhA or CbhB released cellobiose upon incubation with CMC. The presence of D-glucose oligosaccharides larger than cellobiose was probably due to impurities in the enzyme preparations (mainly endoglucanases).

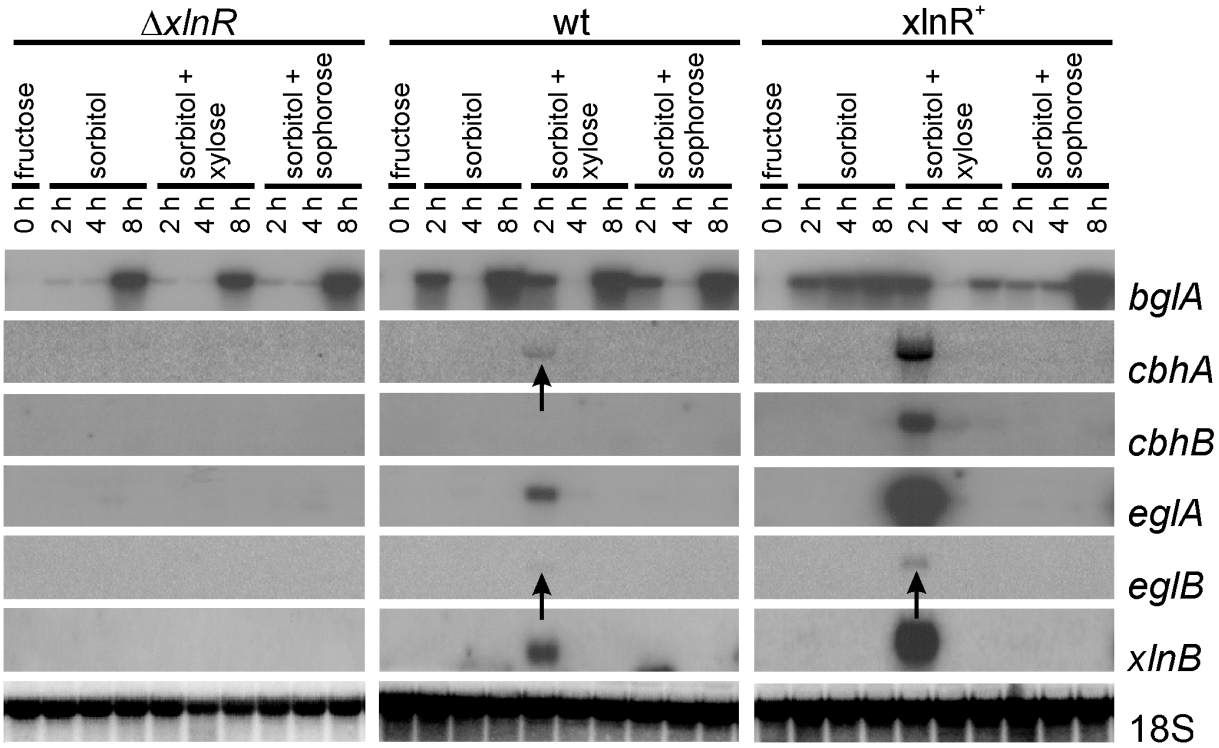

Fig. 3. Northern blot analysis on total RNA of expression of *A. niger* genes encoding cellulose- and xylan-degrading enzymes. Time course induction of *A. niger* NW197 (*ΔxlnR*; *xlnR* deletion mutant), N402 (wt) and N902::pIM230-3.9 (*xlnR*$^{+}$; ten copies of *xlnR*). All three strains were cultured for 18 h in 3 % (w/v) D-fructose and mycelia were subsequently transferred to 25 mM sorbitol, 25 mM sorbitol + 1 mM D-xylose or 25 mM sorbitol + 1 mM sophorose. Blots were hybridised with gene specific probes as indicated and with an 18S rRNA probe as loading control. The arrows indicate low but detectable hybridisation signals.

Both *cbhA* and *cbhB* are expressed in the presence of D-xylose but not of sophorose.

The transcription of five cellulase encoding genes, including the two *cbh* genes, was studied in a transfer experiment with three different strains. The strains used were NW197, a strain in which the xylanolytic transcriptional activator gene *xlnR* is disrupted, N902::pIM230-3.9, which has multiple copies of the *xlnR* gene and the wild-type strain

N402. These three strains were pre-grown on 3% (w/v) D-fructose for 18 h. The mycelium was harvested, washed with minimal medium, and transferred to minimal medium with different carbon sources. After 2, 4 and 8 h, the mycelium was harvested and northern blot analysis was performed with total RNA isolated from the mycelium samples. Although the levels of transcription were low, transcription of both *cbh* genes on D-xylose was observed (Fig. 3). In the *xlnR* multicopy strain, transcription of *cbhA*, *cbhB*, *eglA*, *eglB*, and *xlnB* was visible 2 h after transfer and disappeared 4 h after transfer, probably due to exhaustion of the inducer D-xylose. The transcription of *cbhA* and *cbhB* was also analysed in the wild type which was transferred to 1% (w/v) xylan or 1% (w/v) Avicel cellulose and grown for 24 h. Both *cbh* genes showed higher transcript levels on xylan than on D-xylose, whereas only *cbhB* was transcribed on cellulose (data not shown). The fact that genes are more strongly induced by xylan than by D-xylose has been before [10,26]. It has been shown that although D-xylose induces the transcription of genes controlled by XlnR, the carbon catabolite repressing effect of D-xylose is different from that of xylan. For some of these genes D-xylose displayed repressing properties already at concentrations higher than 1 mM [37]. The patterns of transcription of *cbhA* and *cbhB* resemble those of both of the endoglucanase genes *eglA* and *eglB* and that of the gene encoding endoxylanase B. However, no transcription of *cbhB* was detected in the wild type strain. The *xlnR* disruptant strain was not able to express any of the examined genes except *bglA*, suggesting control of regulation of transcription by the xylanolytic activator XlnR. This control has already been established for the *xlnB, eglA,* and *eglB* genes [28,29]. Addition of sophorose to cultures did not result in an increase in the expression of *bglA*, *cbhA*, *cbhB*, *eglA*, *eglB*, or *xlnB*. The transcription of *bglA* was not specifically induced by D-xylose or sophorose or regulated by XlnR. Thus, although *bglA* is not directly regulated by XlnR, its transcription is indirectly influenced by XlnR. However, the basis of this mechanism is unknown. These findings confirm data obtained earlier [29].

As expected for extracellular enzyme systems under the control of carbon catabolite repression, none of the genes was expressed on D-fructose. The presence of the CreA binding motif in the promoters of *cbhA* and *cbhB*, in combination with the absence of expression of both genes when grown on D-fructose, suggest CreA mediated carbon catabolite repression of *cbhA* and *cbhB*. Similar data where reported for the *cbhI* gene in *A. aculeatus*, which is repressed by D-glucose under inducing conditions [35].

Transcription of *cbhA* and *cbhB* is regulated by the xylanolytic transcriptional activator XlnR.

The data obtained from the northern blot analysis shown in Fig. 2 suggest that, in addition to *eglA* and *eglB*, *cbhA* and *cbhB* are regulated by XlnR. Because of the low transcription levels, this northern blot analysis was repeated with a selection of the samples, which were enriched for poly(A)$^+$ mRNA (Fig. 4). Transcription of *cbhA* was observed in the wild-type, whereas the transcription levels were increased in the *xlnR* multicopy strain. Transcription of *cbhB* was observed only in the *xlnR* multicopy strain, and no transcription of *cbhA* and *cbhB* was observed in the *xlnR* disruptant strain.

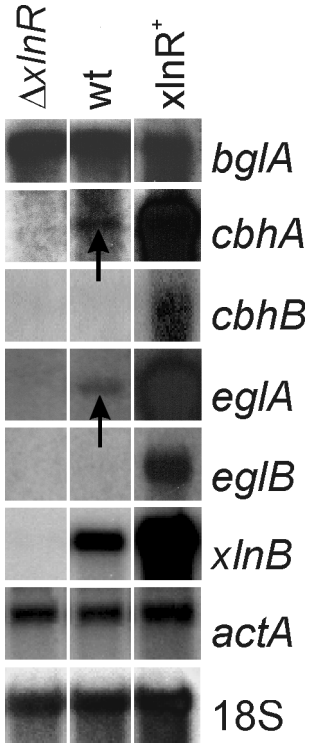

Fig. 4. Northern blot analysis on polyA+ mRNA of expression of *A. niger* genes encoding cellulose- and xylan-degrading enzymes. *A. niger* strains NW197 (*ΔxlnR*), N402 (wt) and N902::pIM230-3.9 (*xlnR*$^+$) were cultured for 18 h in 3 % (w/v) D-fructose and mycelia were subsequently transferred to 25 mM sorbitol + 1 mM D-xylose and grown for 2 h. Blots were hybridized with gene specific probes as indicated and with 18S rRNA and actin (*actA*) probes as loading controls. The arrows indicate low but detectable hybridisation signals.

The effect of additional copies of the *Aspergillus tubingensis xlnA* gene on the expression of *cbhA* and *cbhB*.

The *A. tubingensis xlnA* gene contains three copies of the XlnR binding motif 5'-GGCTAA-3' [28] and strongly titrates XlnR, leading to a decreased expression of other XlnR controlled genes [30]. Similar results have also been obtained with *Aspergillus oryzae* [20]. Northern blot analysis was performed after a transfer experiment with several *A. niger* N902::3*xlnR*-9 strains containing different numbers of *A. tubingensis xlnA* copies integrated into the genome. In this experiment, the *A. niger* strain N902::pIM230-3.9 was chosen as the parental strain because of the elevated levels of transcription of cellulase and xylanase encoding genes. The transcript levels of *cbhA, cbhB, eglA*, and *xlnB* decreased with an increasing number of copies of the *A. tubingensis xlnA* (Fig. 5).

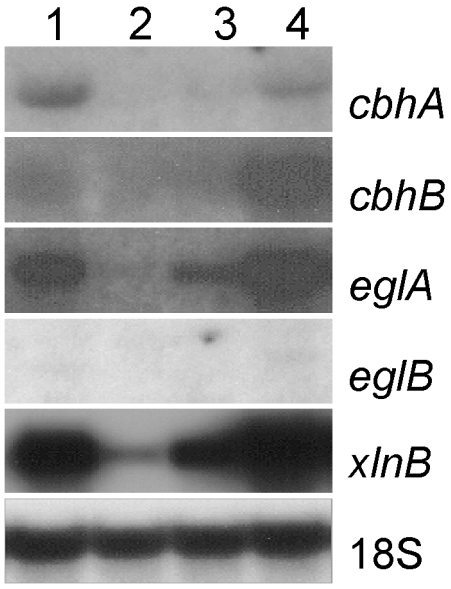

Fig. 5. The effect of additional copies of the *A. tubingensis xlnA* gene integrated into the genome of *A. niger* N902::pIM230-3.(ten copies of *xlnR*)(lane 1) on the transcription of xylan- and cellulose-degrading genes. Strain N902::pIM230::pIM101-6 contains 20 copies of the *xlnA* gene (lane 2), N902::pIM230::pIM101-10 contains 6 *xlnA* copies (lane 3) and N902::pIM230::pIM101-12 contains 2 *xlnA* copies (lane 4). The strains were cultured for 18 h on 3 % (w/v) D-fructose and mycelia were subsequently transferred to 1 % (w/v) D-xylose and grown for 8 h. Blots were hybridized with gene specific probes as indicated and with an 18S rRNA probes as loading control.

DISCUSSION

Two cellobiohydrolase encoding genes from *A. niger* have been isolated and characterised. The *cbhB* gene is not interrupted by introns. The same result was obtained for the *A. aculeatus cbhI* gene [35], whereas all the other fungal *cbhI* genes sequenced, including the *A. niger cbhA* gene, had their structural genes interrupted by introns at various positions. By the classification method based on hydrophobocity analyses of amino acid sequences as proposed by Henrissat and Bairoch [14], both *A. niger* Cbh proteins belong to the glycosyl hydrolases of family 7.

Cellobiohydrolases are composed mostly of three structural domains: a core which contains the hydrolytic site, a Pro/Ser/Thr-rich hinge which protrudes from the catalytic core and tends to be highly glycosylated, and attached to the hinge a highly conserved tail which binds crystalline cellulose [25]. The overall structure of CbhB is similar to those of most other fungal cellobiohydrolases of family 7 in that it contains both the hinge and the conserved CBD at its C-terminus. However, CbhA lacks the CBD and the linker peptide. Covert *et al.* [6] reported that one of the *Panerochaete chrysosporium* genes cellobiohydrolases, namely *cbhI-1*, also consists of a catalytic domain only. The nucleotide sequence downstream of the stop codon of *A. niger cbhA* does not bear resemblance to the conserved CBD in any frame, excluding the possibility of a frame-shift due to sequencing errors. It also has no homology with the region downstream of the stop codon of *P. chrysosporium cbhI-1*. It is now well established that the removal of the CBD has little influence on the activities of cellulases towards soluble substrates while but that it clearly decreases their activity towards insoluble cellulose [25]. It is possible that cellulases with CBDs are required in the early stages of cellulose degradation, when most of the substrate is still insoluble [32]. At later stages, when most of the substrate has been solubilised into oligosaccharides, enzymes without CBDs might be preferred. In *T. reesei* these are generated by proteolysis of the CBD. Apparently, both *A. niger* and *P. chrysosporium* utilize different strategies to achieve the same goal, since both organisms, in contrast to *Trichoderma* species, are able to synthesize cellobiohydrolases with and without a CBD.

A few studies have noted that cellulose and sophorose give rise to the highest levels of cellulase gene expression in *T. reesei* and *Aspergillus terreus* [15,17,26]. The data obtained by the authors of those studies clearly demonstrated the strong inducing power of sophorose when it is added in concentrations of 1 to 2 mM. Sophorose is

therefore regarded as the principal candidate for being the natural inducer of cellulase biosynthesis in *Trichoderma* [17]. Furthermore, the transcription of two endoxylanases (*xyn1* and *xyn2*) and of β-xylosidase (*bxl1*) was also activated when the fungus was cultured on cellulose and, to a lesser level, when it was grown on a mixture of sorbitol and sophorose [26]. Similar results were obtained with *A. terreus* in which cellulose (or derivatives thereof) is able to provoke the biosynthesis of cellulases and xylanases but in which xylan (or derivatives thereof) only induces xylanases [15,16]. Our data suggest an entirely different pattern in *A. niger*. In this fungus the transcription of the two endoglucanases *eglA* and *eglB* and the two cellobiohydrolases *cbhA* and *cbhB* is specifically triggered by D-xylose and not by sophorose. The gene encoding β-glucosidase does not follow this pattern. However, the transcription levels of the cellulase-encoding genes in *A. niger* are less abundant than in *Trichoderma*.

The fact that, besides the xylanolytic genes, four cellulolytic genes are expressed when *A. niger* is grown on D-xylose suggests a common regulatory mechanism controlling the transcription of all these genes. Recently, we demonstrated that the regulation of transcription by XlnR not only directs genes encoding enzymes involved in the degradation of (arabino)xylan but also directs genes encoding two endoglucanases [29]. The transcription pattern of the cellobiohydrolase-encoding gene *cbhA* resembles that of the endoglucanases: no transcription was detected in the *xlnR* disruption mutant, whereas *cbhA* had increased transcription levels in the *xlnR* multicopy strain compared to levels inthe wild-type strain. With *cbhB* we were not able to clearly demonstrate transcription in the presence of D-xylose in the wild-type strain, although, as in *cbhA*, an XlnR binding site (5'-GGCTAA-3') is present in the promoter. It seems that the *cbhB* gene is transcribed at 24 h and later. Transcription of *cbhB* was visible in the wild type strain after being induced by xylan or cellulose for 24 h. Also note that the cDNA clones of *cbhB* were isolated from a xylan-induced cDNA library, which was constructed with RNA isolated 81 and 96 h after inoculation [8]. It is therefore likely that an induction period of 2 h on D-xylose is probably too short to achieve high transcription levels of *cbhB*. In the *xlnR* multicopy strain, however, transcription of both *cbhA* and *cbhB* was evident. Furthermore, introduction of multiple copies of the *A. tubingensis xlnA* gene, which contains three XlnR binding sites, resulted in decreased transcription levels of the *xlnB* gene as well as of all four cellulase-encoding genes. This result suggests the titration of a regulatory factor that all these genes have in common. This regulatory factor

appears to be XlnR activator protein. In *T. reesei*, however, based on results of detailed *in vitro* binding experiments, two adjacent protein binding motifs in the promoter of the *cbh2* gene, which encodes cellobiohydrolase II, were identified. Although a sequence resembling the *A. niger* XlnR binding site was found in the promoter region, based on the results from competition experiments with oligonucleotides derived from the *A. niger xlnD* promoter, it was concluded that the protein that binds to the fragment is not the XlnR homologue in *T. reesei* [39]. This conclusion implies mechanistic differences in the systems of regulation of transcription of genes encoding cellulolytic enzymes in *A. niger* and *T. reesei*.

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Chapter 7

Summarising discussion.

SUMMARISING DISCUSSION

Glycosylhydrolases like amylases, pectinases, arabinanases, cellulases and xylanases are of great interest, not only for their important role in ecological recycling of biomass, but also for their industrial applications. Improved fungal strains which are hyperproductive have been obtained by mutagenesis and selection strategies. Over the last fifteen years many fungal genes encoding glycosylhydrolases were cloned and characterised This has opened the way to study the mechanisms of regulation of gene expression and secretion, and to molecular approaches in strain breeding. When growing in their natural habitat Aspergilli encounter a wide variety of polysaccharides, such as cellulose, pectin, lignin and hemicelluloses as *e.g.* (arabino-)xylan. Since these large molecules cannot be taken up by the fungus directly, Aspergilli secrete a broad range of enzymes that can degrade these complex polymers to monomeric sugars. The research described in this thesis focuses on the molecular genetics of genes involved in the degradation of cellulose and the hemicellulose arabinoxylan.

Chapters 2, 3 and 4 describe studies on the release of L-arabinose residues from arabinoxylan. In Chapter 2, *Aspergillus niger creA* mutants relieved of carbon repression were isolated and the effects of the mutations were studied on the expression of arabinanases and L-arabinose catabolic enzymes. Carbon catabolite repression is a global regulatory mechanism by which in the presence of D-glucose or other rapidly metabolisable carbon sources the expression of genes involved in the utilisation of less-favoured carbon sources is repressed (see for review [27]). The *A. niger creA* mutants were isolated from an *areA* parental strain by selection of colonies that exhibited improved growth on a combination of 4-aminobutanoic acid (GABA) and D-glucose. The *creA* mutants obtained were used to study the involvement of CreA in repression by D-glucose of arabinanases and L-arabinose catabolism in *A. niger*. In the wild-type *A. niger* strain, α-L-arabinofuranosidase A (AbfA), α-L-arabinofuranosidase B (AbfB), endo-arabinanase (AbnA), L-arabinose reductase and L-arabitol dehydrogenase were induced by L-arabinose, but the addition of D-glucose prevented this induction. Repression was relieved to varying degrees in the *creA* mutants, showing that biosynthesis of arabinanases and L-arabinose catabolic enzymes is under control of CreA. The different *A. niger creA* alleles displayed non-hierarchical heterogeneity. This behaviour was also observed in *Aspergillus nidulans* when the effects of several *creA* alleles was studied on

the arabinanase system in this fungus [31]. In fact, the overall patterns of arabinanase expression in both *A. niger* and *A. nidulans* are so similar, that the results from studies conducted with *A. nidulans* can be extrapolated to *A. niger* and *vice versa*.

The latter remark is important in view of the results described in Chapter 3. In this chapter, the cloning, characterisation and disruption of the *A. nidulans abfB* gene is described. The deduced amino acid sequence of the *A. nidulans abfB* gene shows a high identity with other α-L-arabinofuranosidases of family 54 of glycosylhydrolases [10]. Chapter 3 also describes the analysis of expression of *abfB* in an *A. nidulans* wild type strain and several mutant strains. These strains are affected either in their response to ambient pH (*palA1* and *pacC*^{c}*14*), carbon catabolite repression (*creA*^{d}*4*), the ability to utilise L-arabitol as a carbon source (*araA1*) or a combination of both latter mutations. Analysis of *abfB* expression is facilitated in *A. nidulans*, since AbfB is the major α-L-arabinofuranosidase activity found in *A. nidulans* [25]. This in contrast to *A. niger* which has two α-L-arabinofuranosidases, namely AbfA and AbfB [32]. *p*-Nitrophenyl-α-L-arabinofuranoside (pNP-A) activities determined in the culture medium can thus be correlated to the transcription level of *abfB*. Furthermore, the existence of some well defined mutants gives an excellent tool to investigate the effect of several alleles on the expression of the *abfB* gene in *A. nidulans*. The accumulation of *abfB* mRNA, total α-L-arabinofuranosidase activity and AbfB protein levels have been determined in these strains. The data obtained clearly demonstrated a super-induction of AbfB expression in the *araA1*/*creA*^{d}*4* double mutant, *i.e.* the effect of the combination of both alleles on the expression of AbfB was higher than the sum of the alleles separately. This indicates that both the accumulation of inducer and relieve of repression of transcription, in this case carbon catabolite repression, needs consideration when designing strain improvement strategies.

Although disruption of the *A. nidulans abfB* gene demonstrated that this gene codes for the major α-L-arabinofuranosidase, also other pNP-A hydrolysing activities are expressed in this fungus. Based on immunological data, it was concluded that one of those minor activities appeared to be an enzyme similar to *A. niger*/*A. tubingensis* arabinoxylan arabinofuranohydrolase A (AxhA)(see also Chapter 4).

Chapter 4 describes the isolation and characterisation of the arabinoxylan-arabinofuranohydrolase A (AxhA) encoding genes from the closely related fungi *A. niger* and *A. tubingensis*. The primary structure of these enzymes is not related to those of other

α-L-arabinofuranosidases, except for a low, but significant similarity with XylC, a bacterial α-L-arabinofuranosidase from *Pseudomonas fluorescens* subsp. *cellulosa* which acts only on oat spelt xylan. Recently, both *Aspergillus* AxhA proteins have been included in family 62 of glycosyl hydrolases [10]. This family also includes *P. fluorescence* XylC and two α-L-arabinofuranosidases from *Streptomyces* species [26,33]. All the enzymes classified in this family are L-arabinose releasing activities which act specifically on (arabino-)xylan. These proteins were assigned to this family on the basis of the similarity of their catalytic domains. However, the AxhA enzymes consist only of a catalytic domain, whereas *P. fluorescence* XylC contains also a cellulose binding domain (CBD) and both *Streptomyces* proteins contain a xylan binding domain (XBD) instead. The primary structure of the substrate-binding domains, CBD and XBD respectively, are clearly distinct [33]. This explains why XylC binds specifically to Avicel and not to xylan [15], whereas *S. lividans* AbfB binds specifically to insoluble xylan [33].

AxhA was first isolated and described by Kormelink *et al.* [19]. The enzyme releases 1,2-α- and 1,3-α-linked arabinofuranosyl groups from arabinoxylans and arabinoxylan-derived oligosaccharides but not from other L-arabinose-containing substrates. Moreover, AxhA shows a very low activity towards artificial substrate (pNP-A)[18,19]. This in contrast to AbfB which also displays arabinose-releasing activity towards L-arabinan, arabinogalactan and 1,5-α-linked α-arabinofuranosyl oligosaccharides and is active towards pNP-A [32]. The specific activity of *A. tubingensis* AxhA on pNP-A is approximately 15 x 10^{-3} U mg^{-1} versus 23.5 U mg^{-1} for AbfB [6]. Apparently, the nature of the group to which the α-L-arabinosyl residue is attached is more important for the activity of AxhA than of AbfB. Although both AxhA and AbfB are active on polymeric arabinoxylan, AxhA removes the L-arabinosyl residues more efficiently. This is reflected in plate assays with 5% D-glucose + 1% oat spelt xylan comparing *A. niger* transformants harbouring expression constructs in which the *axhA* or the *abfB* gene is driven by the *A. niger pkiA* promoter. AxhA producing strains could be selected on the basis of a halo of precipitated xylan appearing around the colony. By the removal of most of the L-arabinose side residues, the xylan chains can form hydrogen bonds in the absence of steric hindrance by the α-L-arabinosyl residues and therefore precipitate. None of the AbfB transformants caused any halo suggesting a less efficient removal of the L-arabinosyl residues from the xylan backbone [6,19]. Kinetic

experiments demonstrated that AxhA is able to release L-arabinosyl substituents from terminal, as well as from non-terminal, single substituted D-xylopyranosyl residues in low molecular weight oligosaccharides, whereas AbfB can only release L-arabinosyl substituents from terminal single substituted D-xylose units [18]. Besides this difference in mode of action, AxhA and AbfB differ also in their stereochemical course the hydrolysis. Whereas AbfB, like many other exo-glycanases, acts by retaining of anomeric sugar configuration, AxhA is tentatively classified as an inverting enzyme, like many other glycosidases and endo-glycanases [24]. If also the sizes of both enzymes are taken into account, it is tempting to suggest a different manner in which the α-L-arabinofuranosyl residues are released from the D-xylose backbone by both enzymes. AbfB, which has a molecular weight of approx. 65 kDa, shows a true exo-type mechanism: it encloses the L-arabinofuranosyl residue and hydrolyses the linkage between the L-arabinofuranosyl residue and the xylose backbone. Due to its relative small size of 32 kDa, AxhA is able to squeeze itself down to the arabinose-xylan linkage and hydrolyses this linkage in an endo-type fashion.

Chapter 4 also describes the transcriptional analysis of the *axhA* gene in *A. niger*. Northern blot analysis demonstrated that the expression of *axhA* was regulated at the transcriptional level. Transcription of *axhA* was derepressed in *creA*^{d} mutants and carbon catabolite repressed by D-glucose. Furthermore, the *axhA* expression pattern differed from that of *abfB,* since the former gene was strongly induced by birchwood xylan and much less by L-arabitol or L-arabinose, which were the strongest inducers of *abfB* transcription. Although both AxhA and AbfB are active on arabinoxylan, the expression of these two L-arabinose releasing activities is regulated differently. From these experiments it was concluded that the transcription of *axhA* is possibly regulated in a similar manner to that proposed for the expression of *xlnA* in *A. tubingensis* [7]. For *xlnA* gene expression the presence of a specific transcriptional activator was demonstrated. This activator was proposed to stimulate the transcription other structural xylanolytic genes as well. The transcriptional analysis data described in Chapters 2 and 4 demonstrate that both *abfB* and *axhA* are subjected to carbon catabolite repression in *A. niger*. Both genes are also supposed to be regulated by a route-specific transcriptional activator protein [5](Chapters 2, 4 and 5). However, it is most likely that *abfB* and *axhA* are regulated by different route-specific transcriptional activator proteins. This was

concluded after comparison of the transcription patterns of both genes under inducing conditions in the presence D-glucose in *creA*^{d} genetic backgrounds.

The same conclusion is drawn in Chapter 5 which clearly demonstrates that *axhA* is transcriptionally regulated by XlnR, whereas *abfB* is not. The *xlnR* gene, encoding a transcriptional activator co-ordinating xylanolytic expression in *A. niger*, was isolated by complementation of an *A. niger* mutant lacking xylanolytic activity [23]. The XlnR protein consists of 875 amino acids capable of forming a zinc binuclear cluster domain with similarity to the zinc clusters of the GAL4 superfamily of transcription factors. The XlnR-binding site 5'-GGCTAA-3' is also found within several xylanolytic promoters of various *Aspergillus* species, *Trichoderma reesei* and *Penicillium chrysogenum*. Chapter 5 gives a more detailed transcriptional analysis of genes encoding enzymes involved in xylan degradation and two endoglucanases involved in cellulose degradation in *A. niger*. A strain with a loss-of-function mutation in the *xlnR* gene, a strain with multiple copies of this gene and a wild-type strain were investigated in order to define which genes are controlled by XlnR. The data presented 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 β-xylosidase, respectively). Also, the transcription of the genes encoding the accessory enzymes involved in xylan degradation, including α-glucuronidase A (*aguA*), acetylxylan esterase A (*axeA*), arabinoxylan arabinofuranohydrolase A (*axhA*) and feruloyl esterase A (*faeA*), were found to be controlled by XlnR. In addition to the genes already mentioned, XlnR is involved in the regulation of transcription of two cellobiohydrolase encoding genes (*cbhA* and *cbhB*; see chapter 6), an α-galactosidase and a β-galactosidase encoding gene (*aglB* and *lacA*, respectively)[34].AglB and LacA are both expressed when grown on arabinoxylan or D-xylose and are implicated to be involved in the degradation of hemicelluloses, including arabinoxylan, and pectin [34]. These results indicate a key role of the xylanolytic regulator XlnR in the degradation of hemicellulose and cellulose.

The fact that XlnR also regulates the transcription of two genes encoding EglA and EglB encouraged us to study the transcriptional regulation of two cellobiohydrolase genes, *cbhA* and *cbhB*. This research is described in Chapter 6. cDNA clones of both genes were isolated from a xylan-induced cDNA library of *A. niger*. The cDNA clones were subsequently used to isolate the corresponding genes from an *A. niger* genomic library. An unexpected result was that *cbhA* and *cbhB* are both induced by D-xylose and

not by sophorose. However, the transcription of cellulase-encoding genes in *A. niger* is less pronounced compared to *T. reesei*. The induction of *cbhA*, *cbhB*, *eglA* and *eglB* by D-xylose is differenr to what is generally found in *T. reesei* [14,21]. There it was found that when a wild-type *T. reesei* strain was grown on cellulose or sorbitol + sophorose, the gene encoding cellobiohydrolase 1 (*cbh1*) was strongly induced and the genes encoding β-xylosidase (*bxl1*) and two endoxylanases (*xyn1* and *xyn2*) were moderately induced. However, when grown on oat spelt xylan *bxl1*, *xyn1* and *xyn2* were more induced, while *cbh1* was only poorly expressed. No expression of any of these genes was found when grown on 5% D-xylose, although the authors suggest that the high D-xylose concentration probably had a repressive effect [21]. Growth on sorbitol + xylobiose resulted in a poor induction of *bxl1* and *xyn2* and no induction of *cbh1*. Specificity of cellulase and xylanase induction was already demonstrated in *T. reesei* [12] and similar results were obtained when *T. reesei* was cultivated on media based on cellulose and xylan as main carbon source [1]. Also in *Aspergillus terreus*, biosynthesis of cellulose- and xylan-degrading enzymes appears to be under separate regulatory control [13]. Later, the effect of homo- and heterodisaccharides composed of D-glucose and D-xylose on the induction of cellulose- and xylan-degrading enzyme systems in *A. terreus* was studied in more detail [11]. This showed that the heterodisaccharide 2-*O*-β-D-glucopyranosyl D-xylose (Glc-β1-2Xyl) was the most powerful inducer of both cellulolytic and xylanolytic enzymes. Sophorose only induced cellulases, whereas xylobiose induced the biosynthesis of xylanases and only poorly that of cellulases. These findings support the concept of separate regulatory control of the synthesis of cellulases and xylanases in *A. terreus*. Hrmová et al. [11] proposed an important role for mixed disaccharides, composed of D-glucose and D-xylose moieties, which may occur in nature, in regulating the synthesis of wood-degrading enzymes. But as indicated by the authors, the group of heterodisaccharides examined cannot be regarded as universal inducers of cellulases and xylanases in fungi, since these compounds were ineffective in *T. reesei*. In *Aspergillus aculeatus* F50, which is related to *A. niger*, synthesis of cellulases was most pronounced when the fungus was cultured on (arabino-)xylan. The levels of cellulase activities in the medium was approx. 2-5 fold compared to cultures with cellulose-based media [22].

From the literature reports cited above, it may be concluded that fungi interact with their environment in their own specific way and that the underlying mechanisms of gene expression can be different from organism to organism. When comparing the data

described in this thesis with data obtained in *T. reesei* [12,14,21], it is clear that the mechanisms of induction of cellulase- and xylan-degrading enzymes in *T. reesei* and *A. niger* are significantly different. This makes it interesting to compare these two fungi in relation to the regulation of the gene expression of these enzyme systems.

The data presented in Chapter 6 show that the xylanolytic transcriptional activator XlnR is involved in the regulation of transcription of the *A. niger* cellobiohydrolase genes *cbhA* and *cbhB*. The fact that in addition to xylanolytic genes, also four cellulolytic genes are expressed when grown on D-xylose suggests a common regulation mechanism controlling the expression of these genes. The pattern of transcription of *cbhA* resembles those of *eglA* and *eglB*. No expression was detected in the *xlnR* disruption mutant, whereas *cbhA* had increased transcription levels in the *xlnR* multicopy strain compared to the wild-type strain. In the case of *cbhB* we were not able to clearly demonstrate transcription in the wild-type strain in the presence of D-xylose. It seems that the *cbhB* gene is transcribed late during growth under induction conditions and it is therefore likely that level of *cbhB* transcription was sub-optimal under the conditions tested. In the *xlnR* multicopy strain, however, transcription of both *cbhA* and *cbhB* is evident. The presence of the XlnR binding site 5'-GGCTAA-3' in the promoters of the xylanolytic genes, *eglA*, *eglB* and both *cbhA* and *cbhB* genes supports the findings obtained by northern blot analysis that XlnR is involved in the activation of transcription of both *cbh* genes. Furthermore, the introduction of multiple copies of the *A. tubingensis xlnA* gene, which contains three XlnR binding sites [7,23], resulted in decreased transcription levels of the *xlnB* gene as well as of all four cellulase-encoding genes. This suggests titration of the regulatory factor XlnR which all these genes have in common. Titration of a common transcriptional regulatory factor involved in the expression of genes encoding xylanolytic enzymes was also demonstrated in *Aspergillus oryzae*. Introduction of 64 copies of the promoter region of the endoxylanase encoding gene *xynF1* in the same direction led to reduced expression of both xylanase and β-xylosidase genes in the transformants [16]. Recently, two adjacent protein binding motifs acting co-operatively in the induction by cellulose were identified in the *cbh2* (cellobiohydrolase II-encoding) promoter of *T. reesei*. The nucleotide sequence 5'-ATTGGGTAATA-3', designated as *cbh2*-activating element (CAE), is responsible for binding of protein complexes from cellulase-forming (induced) and non-induced mycelia and is essential for induction of gene expression by cellulose and sophorose *in vivo* [35]. The CCAAT (=ATTGG) motif is recognised by a *T.*

reesei homologue of Hap3, the GTAATA motif probably interacts with the specific transcriptional activator. Since the 3' area bears some resemblance to the *A. niger* XlnR binding site, a *T. reesei* homologue of XlnR might be this transcriptional activator. However, based on the results from competition experiments using oligonucleotides derived from the *A. niger xlnD* promoter, it was concluded that the protein binding to the fragment is not the XlnR homologue of *T. reesei* [35]. Furthermore, two putative regulatory genes *ace1* and *ace2* (Activator of Cellulase Expression) of *cbh1* were isolated from *T. reesei*. The *ace2* gene product, ACE II, contains a zinc binuclear DNA-binding domain and was reported to bind to the DNA sequence 5'-GGCTAATAA-3', but it is less than half the size of *A. niger* XlnR [28,35]. Thus, it was speculated that ACE II and a complex containing Hap3 are involved in *cbh2* gene transcription [35].

Using the classification method based on hydrophobic cluster analysis as proposed by Henrissat and Bairoch [10], both *A. niger* CbhA and CbhB proteins may be included in the glycosyl hydrolases of family 7. CbhA showed the highest identity with CbhB (65.3%) and CbhI from *Penicillium janthinellum* (62.9%). CbhB showed the highest identity with CbhI from *A. aculeatus* (72.6%). All members of this family are cellobiohydrolases of fungal origin and are mostly composed of three structural domains: a core which contains the hydrolytic site, a Pro/Ser/Thr-rich hinge which protrudes the catalytic core and tends to be highly glycosylated, and attached to the hinge a highly conserved tail which binds crystalline cellulose [20]. The deduced amino acid sequence of CbhB is similar to that of most other cellobiohydrolases of family 7 in that it contains the conserved CBD at its C-terminal end attached to the catalytic domain via a Ser/Thr-rich linker peptide. The CBD does not only decrease the effective K_m of the enzyme with natural substrates but also releases cellulose chains from the cellulose crystal prior to hydrolysis by the catalytic domain [17]. It is now well established that the removal of the CBD has little effect on the activity of cellulases towards soluble substrates while their activity towards insoluble cellulose is clearly decreased [20]. The proteolytic removal of the linker peptide and the CBD may serve as an *in vivo* mechanism to alter the properties of cellulases during hydrolysis, when complex insoluble substrates are gradually shortened to soluble and more accessible substrates [29]. However, CbhA lacks a CBD and a linker peptide and only consists of a catalytic domain. A similar gene organisation was found in the white-rot fungus *Phanerochaete chrysosporium* [4]. Apparently, both *A. niger* and *P. chrysosporium* utilise a different strategy to achieve the same goal, since

both organisms, in contrast to *Trichoderma* species, are able to produce cellobiohydrolases with and without a CBD. Recently, the isolation of the gene (*exo1*) and characterisation of the enzymatic properties of a major exoglucanase of the fungus *Humicola grisea* without a cellulose-binding domain was reported [30]. Exo1 is 45 % and 47.5 % identical to *A. niger* CbhA and CbhB, respectively. The transcription levels of *exo1* and *cbh1*, encoding a cellobiohydrolase with a CBD, are high on Avicel and repressed by D-glucose. The addition of a CBD and a hinge region to Exo1 caused a decrease in its enzymatic activity. However, detailed enzyme activity studies suggested that Exo1 is not a cellobiohydrolase but rather an exoglucanase hydrolysing cellulosic substrates by releasing exowise D-glucose units.

Cellulose and hemicelluloses, for example arabinoxylan, and lignin are the major polymeric constituents of plant cell walls and form the largest reservoir of fixed carbon in nature. Degradation of these polysaccharides is an important process in recycling carbohydrates from plant cell walls. Microorganisms play a crucial role in this recycling process, due to their capacity to secrete a wide range of polysaccharide degrading enzymes. The plant cell walls are composed of complex polysaccharides and structural proteins that interlace long, crystalline ribbons of cellulose spooled around each cell in several strata. The two most abundant structural polysaccharides are cellulose and the hemicellulose xylan, which are closely associated in the plant cell walls [3]. All the extracellular polysaccharide hydrolases from *P. fluorescence* contain a CBD which are required for efficient hydrolysis of xylan and cellulose [9]. In the normal environment of soil saprophytes, where efficient degradation of plant structural polysaccharides demands prolonged association of active enzymes with relatively recalcitrant substrates, CBDs would confer a selective advantage on plant-cell-wall-degrading enzymes by promoting intimate contact between enzyme and substrate. It was therefore proposed that the diversity of xylan structure coupled with the high concentration of cellulose in different plant species could be the basis for the evolution of a single CBD, rather than protein domains which bind to each plant cell wall polysaccharide [15]. However, *S. lividans* AbfB has a binding domain binding specifically to xylan and most other α-L-arabinofuranosidases, *e.g.* AxhA, lack any binding domains. This suggest that the molecular architecture of the *P. fluorescence* polysaccharide degrading system is not a common feature, but rather a strategy to degrade plant cell wall material.

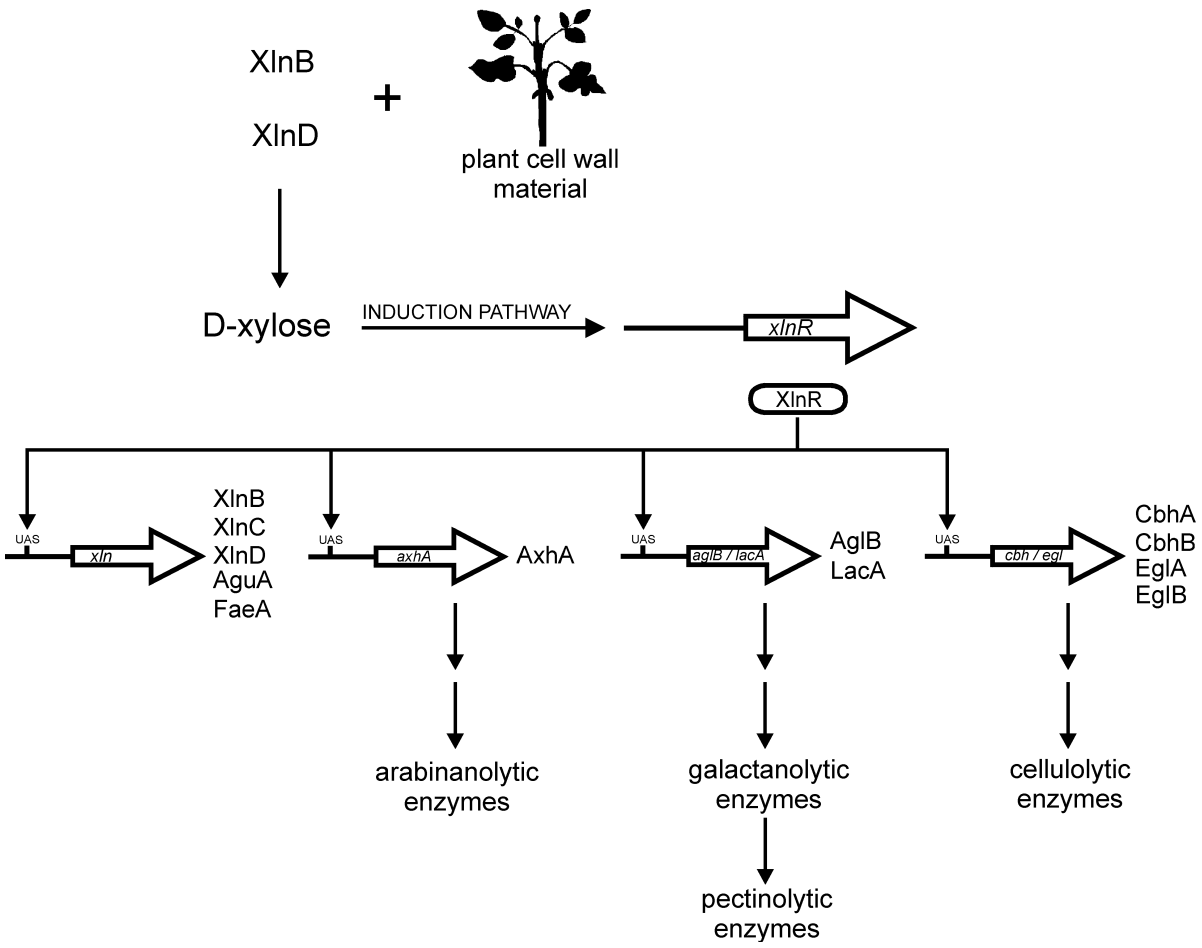

Fig. 1: Schematic model for the regulation of genes encoding enzymes involved in the degradation of plant cell wall polysaccharides in *A. niger*. The model presumes that low levels of endoxylanase B (XlnB) and β-xylosidase (XlnD) liberate small amounts of D-xylose from the cellulose-hemicellulose matrix of the plant cell wall. XlnR, which is induced by the presence of D-xylose, activates apart from genes encoding xylanolytic also a number of genes involved in the release of monosaccharides other than D-xylose. These monosaccharides then induce the expression of genes which are not directly controlled by XlnR, *e.g.* the arabinanases encoding genes *abfA*, *abfB* and *abnA*, or genes encoding pectinolytic enzymes. These enzymes are directly or indirectly involved in the degradation of other polysaccharides present in the wall matrix.

The data described in this thesis suggests a different strategy employed by *A. niger*. The genes *xlnB* and *xlnD*, encoding endoxylanase B and β-xylosidase respectively, are expressed at a low basal level [8]. This would be analogous to what is observed in *T. reesei*. Transcripts of two members of the cellulase system, *cbh1* and *egl1*, are present at low basal levels in non-induced conditions. This basal cellulase activity would digest cellulose, which releases oligosaccharides that are able to enter the cell and trigger

expression of all cellulases. The transcript levels of *cbh1* and *egl1* are induced at least 1100-fold in the presence of cellulose [2].

In *A. niger* xylanase B and β-xylosidase, encoded by *xlnB* and *xlnD* respectively, would be present at low basal levels in the uninduced fungus or alternatively due to C-limitation these genes might be the first ones that become derepressed [8]. These enzymes liberate D-xylose which then enters the cell and induces the xylanolytic genes via the xylanolytic transcriptional activator protein XlnR (Fig. 1). However, this would not only trigger the induction of the main xylanolytic enzymes, but also the induction of accessory enzymes (AguA, AxeA, AxhA and FaeA), cellulolytic enzymes (CbhA, CbhB, EglA and EglB) and enzymes able to liberate D-galactosyl residues from polysaccharides (α-galactosidase B (AglB) and β-galactosidase A (LacA))[34]. These enzymes are then able to release other mono- or oligosaccharides being able to induce the expression of other polysaccharide-degrading enzymes. For example, the two cellulases and two endoglucanases regulated by XlnR will release sugar compounds that induce the complete cellulolytic system. AxhA releases L-arabinose being the inducer of the arabinan-degrading enzyme system [32] and the galactosidases will induce the galactan-degrading system. Moreover, the β-galactosidase LacA is also involved in pectin degradation [34] and might therefore be involved in triggering the expression of pectin degrading enzymes as well. According to this model, plant-cell-wall-degrading enzymes would be induced in a cascade enabling the fungus to interact in a fast and efficient manner with its environment.

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Samenvatting

Glycosylhydrolases, zoals bijv. cellulases en xylanases, zijn ecologisch belangrijk door hun rol in het recyclen van biomassa. Bovendien is er een groeiende interesse voor deze enzymen vanwege hun potentie in industriële applicaties. In hun natuurlijke omgeving komen Aspergilli allerlei polysacchariden tegen zoals cellulose, hemicelluloses (bijv. arabinoxylan) en pectine. Deze grote moleculen kunnen niet direct opgenomen worden door de schimmel, maar moeten eerst in kleinere stukken afgebroken worden. Aspergilli zijn in staat om een grote verscheidenheid aan enzymen uit te scheiden die deze complexe polymeren op kunnen splitsen in momomere suikers. Deze suikers worden vervolgens opgenomen door de schimmel en fungeren dan als koolstofbron. Het in dit proefschrift beschreven onderzoek concentreert zich op de moleculaire genetica van genen die betrokken zijn bij de afbraak van cellulose en de hemicellulose arabinoxylan.

In hoofdstukken 2, 3 en 4 staat de afsplitsing van L-arabinose residuen van arabinoxylan centraal. Hoofdstuk 2 beschrijft de isolatie van *Aspergillus niger creA* mutanten en het effect van deze mutaties op de expressie van arabinanases en L-arabinose katabole enzymen. Koolstof kataboliet repressie is een algemeen regulatie mechanisme waarbij de aanwezigheid van D-glucose of een andere snel metaboliseerbare koolstofbron de expressie remt van genen die betrokken zijn bij de utilisatie van minder snel metaboliseerbare koolstofbronnen. In *Aspergillus* speelt het repressor eiwit CreA een belangrijke rol in koolstof repressie. De in dit hoofdstuk beschreven *creA* mutanten vertoonden allen koolstof derepressie, d.w.z. dat arabinanases en L-arabinose katabole enzymen ook in aanwezigheid van D-glucose tot expressie gebracht worden. De *A. niger creA* mutanten werden geïsoleerd uit een *areA* ouderstam door kolonies te selecteren die beter groeiden op de combinatie 4-aminobutaanzuur (GABA) en D-glucose. De verkregen *creA* mutanten werden gebruikt om de betrokkenheid van CreA in de repressie van D-glucose op de expressie van arabinanases en L-arabinose katabolisme beter te bestuderen. In de *A. niger* wild type stam werd de expressie van α-L-arabinofuranosisdase A (AbfA), α-L-arabinofuranosidase B (AbfB), endo-arabinanase A (AbnA), L-arabinose reductase en L-arabitol dehydrogenase geïnduceerd door L-arabinose, maar toevoeging van D-glucose remde de biosynthese van deze enzymen. In de *creA* mutanten werd de repressie door D-glucose in verschillende mate opgeheven, hetgeen wijst op controle de biosynthese van arabinanases en L-arabinose katabole enzymen door CreA.

Verschillende studies laten zien dat de biosynthese hemicellulases in zowel *A. niger* als *A. nidulans* op vergelijkbare wijze worden gereguleerd. Dit maakt het mogelijk om resultaten van vergelijkbare studies verricht met *A. nidulans* te extrapoleren naar *A. niger*. Dat laatste is belangrijk voor de resultaten beschreven in hoofdstuk 3. In dit hoofdstuk wordt de klonering, karakterisering en disruptie van het *A. nidulans abfB* gen beschreven. De afgeleide aminozuur sequentie van AbfB vertoont een hoge mate van homologie met α-L-arabinofuranosidases uit andere schimmels die zijn ingedeeld in familie 54 van glycosylhydrolases. Tevens werd de expressie van *abfB* geanalyseerd in een *A. nidulans* wild type stam en een aantal mutanten. De mutaties in deze stammen beïnvloeden onder andere de reactie op veranderingen van de externe pH (*palA1* en *pacC*^{c}*14*), de koolstof kataboliet repressie (*creA*^{d}*4*) of het vermogen om L-arabitol als koolstofbron te gebruiken (*araA1*). Doordat er goed gedefinieerde mutanten van *A. nidulans* beschikbaar zijn is deze schimmel bij uitstek geschikt om het effect te bestuderen van verschillende allelen op de *abfB* expressie. In de wild type stam en mutanten werden de accumulatie van *abfB* mRNA, de totale α-L-arabinofuranosidase activiteit en het AbfB eiwitgehalte bepaald. De verkregen data lieten duidelijk zien dat de expressie van *abfB* transcriptioneel gereguleerd is. Het transcriptie niveau van *abfB* was verhoogd in de *palA1* stam terwijl de expressie werd gerepresseerd in de *pacC*^{c}*14* stam, hetgeen suggereert dat AbfB tot expressie wordt gebracht bij een zure externe pH. De expressie van AbfB werd super-geinduceerd door de ophoping van de inducer in een koolstof gederepresseerde genetisch achtergrond in de *araA1*/*creA*^{d}*4* dubbelmutant. Met andere woorden, het effect van de combinatie van beide mutaties op de expressie is groter dan de som van allelen apart. Gendisruptie van *abfB* in *A. nidulans* toont aan dat dit gen inderdaad codeert voor de voornaamste α-L-arabinofuranosidase activiteit. Daarnaast werden er ook andere α-L-arabinofuranosidase activiteiten gevonden in deze schimmel. Mede gebaseerd op immunologische data werd geconcludeerd dat één van die activiteiten veroorzaakt word door een enzym dat overeenkomstig is met arabinoxylan-arabinofuranohydrolase A (AxhA) van *A. niger/Aspergillus tubingensis* (zie ook hoofdstuk 4).

Hoofdstuk 4 beschrijft de isolatie en karakterisatie van de genen die coderen voor arabinoxylan-arabinofuranohydrolase A (AxhA) van de nauw verwante schimmels *A. niger* en *A. tubingensis*. AxhA is alleen actief op arabinoxylan en is in staat om de L-arabinose zijgroepen af te splitsen van de xylan hoofdketen. De aminozuur sequenties van deze genen vertoonden geen verwantschap met α-L-arabinofuranosidases, behalve

met XylC. XylC is een bacteriële α-L-arabinofuranosidase uit *Pseudomonas fluorescens* subsp. *cellulosa* dat eveneens alleen actief is op xylan. Onlangs zijn beide AxhA eiwitten en XylC van *P. fluorescens* ingedeeld in familie 62 van de glycosyl hydrolases. Voor deze indeling, gebaseerd op hydrofobe cluster analyse, werd de afgeleide aminozuur sequentie van de katalytische domeinen van de enzymen gebruikt. De AxhA enzymen bevatten namelijk geen specifiek substraat bindings domein, dit in tegenstelling tot de bacteriële enzymen die tot deze familie behoren.

De expressie van *axhA* wordt gereguleerd op transcriptioneel niveau. De transcriptie van *axhA* werd gederepresseerd in *creA*^{d} mutanten en koolstof gerepresseerd door D-glucose. Uit northern blot analyses bleek dat de transcriptie van *axhA* door andere koolstofbronnen werd geïnduceerd dan de transcriptie van *abfB*. Terwijl *axhA* het sterkst geïnduceerd werd door xylan, en in mindere mate door L-arabitol en L-arabinose, zijn deze laatste twee suikers juist de sterkste inducers voor *abfB* transcriptie. Ondanks dat zowel AbfB als AxhA L-arabinose groepen van xylan kunnen verwijderen worden beide corresponderende genen dus op verschillende wijze op het niveau van transcriptie gereguleerd.

De resultaten beschreven in hoofdstuk 5 bevestigen deze conclusie nog eens. Het *axhA* gen wordt duidelijk transcriptioneel gereguleerd door XlnR, terwijl dit niet het geval is voor *abfB*. XlnR is een activator eiwit dat de transcriptie activeert van xylanolytische genen. Het is een DNA-bindend eiwit en herkent het sequentie element 5'-GGCTAA-3'. Dit sequentie motief is gevonden in een aantal promoters van xylanolytische genen in verschillende *Aspergillus* soorten, *Trichoderma reesei* en *Penicillium chrysogenum*. Hoofdstuk 5 geeft een meer gedetailleerde transcriptie analyse in *A. niger* van genen die coderen voor enzymen betrokken bij de afbraak van xylan en twee endoglucanases die betrokken zijn bij de afbraak van cellulose. Om te onderzoeken welke van deze genen gereguleerd worden door XlnR werd gebruik gemaakt van een stam met een loss-of-function mutatie in het *xlnR* gen, een stam met extra kopieën van dit gen en een wild type stam. De data laten zien dat XlnR de transcriptie reguleert van *xlnB, xlnC* en *xlnD* welke coderen voor de xylanolytische enzymen die inwerken op de xylan hoofdketen (respectievelijk, endoxylanases B en C en β-xylosidase). Ook reguleert XlnR de transcriptie van genen die coderen voor enzymen die zij-groepen verwijderen van de xylan hoofdketen, zoals α-glucuronidase A (*aguA*), acetylxylanesterase A (*axeA*), arabinoxylan arabinofuranohydrolase A (*axhA*) en feruloylesterase A (*faeA*). Bovendien

worden ook de genen die coderen voor twee endoglucanases, namelijk *eglA* en *eglB*, gereguleerd door XlnR.

Het feit dat XlnR de transcriptie reguleert van twee endoglucanases bewoog ons ertoe om deze studie uit te breiden naar twee andere genen die betrokken zijn bij de afbraak van cellulose, namelijk de genen coderend voor de cellobiohydrolases CbhA en CbhB. Deze resultaten zijn beschreven in hoofdstuk 6. cDNA klonen van beide genen werden geïsoleerd uit een xylan-geinduceerde cDNA bank van *A. niger*. Deze cDNA klonen werden vervolgens gebruikt om de corresponderende genen te isoleren. In de literatuur wordt sophorose vaak aangeduid als de belangrijkste inducer van genen die coderen voor cellulolytische enzymen. De resultaten beschreven in hoofdstuk 6 laten echter zien dat in *A. niger* de transcriptie van beide *cbh* genen geïnduceerd worden door D-xylose en niet door sophorose. De expressieniveaus in *A. niger* liggen echter wel lager dan die gerapporteerd voor bijv. *T. reesei* wat een goede producent is van cellulolytische enzymen. In *A. niger* worden *cbhA* en *cbhB* transcriptioneel gereguleerd door XlnR. De regulatie van de transcriptie van beide *cbh* genen vertoont grote gelijkenis met die van andere xylanolytische genen en de twee voor endoglucanase coderende genen *eglA* en *eglB*, d.w.z. ten opzichte van de wild type stam geen detecteerbare transcriptieniveaus in de stam waarin het *xlnR* gen uitgeschakeld is, en verhoogde expressie in de *xlnR* multicopy stam. Ook bleek het niveau van transcriptie van deze genen gerelateerd aan het aantal kopieën van het *A. tubingensis* endoxylanase A gen (*xlnA*) dat geïntegreerd was in het *A. niger* genoom. De promoter van *xlnA* bevat 3 XlnR bindingsplaatsen en komt niet voor in *A. niger*. De introductie van meerdere *xlnA* genen in het *A. niger* genoom resulteerde in verlaagde transcriptieniveau's voor *cbhA* en *cbhB*. Deze resultaten suggereren titratie van een regulatie factor die al deze genen gemeen hebben. Deze factor blijkt XlnR te zijn.

Volgens de classificatie methode zoals voorgesteld door Henrissat en Biaroch worden zowel *A. niger* CbhA als CbhB ingedeeld in familie 7 voor glycosyl hydrolases. Vergelijking van aminozuur sequenties liet zien dat CbhA het meest leek op CbhB (65,3% identiek) en CbhI van *P. janthinellum* (62,9%). CbhB vertoonde de hoogste homologie met CbhI van *A. aculeatus* (72,6% identiek). Vrijwel alle enzymen uit familie 7 zijn cellobiohydrolases uit schimmels en zijn meestal opgebouwd uit 3 structurele domeinen: een katalytisch domein, een Pro/Ser/Thr-rijke linker-peptide die vaak sterk geglycosyleerd is en een cellulose bindingsdomein (CBD). Het CBD is een domein dat aan kristallijn cellulose kan binden. Dit CBD is met name belangrijk wanneer

onoplosbare cellulose afgebroken moet worden omdat het de effectieve bindingsconstante (K_m) van het eiwit voor het substraat verlaagt. Wanneer het substraat oplosbaar is zijn de CBDs minder van belang omdat de katalytische domeinen nu een betere toegang tot het substraat hebben. De CBD wordt onder deze condities vaak proteolytisch afgesplitst. CbhB heeft net als vele andere cellobiohydrolases eveneens een CBD. CbhA heeft echter geen linker-peptide noch een CBD. Het feit dat *A. niger* zowel een cellobiohydrolase met een CBD alsmede een cellobiohydrolase zonder CBD tot expressie kan brengen suggereert dat deze schimmel zich in de evolutie een andere strategie heeft aangemeten.

Zoals al eerder opgemerkt bestaat de celwand van planten uit een complex netwerk van cellulose, hemicelluloses (bijv. arabinoxylan) en pectine. Micro-organismen, waaronder schimmels, zijn belangrijk omdat ze in staat zijn een grote verscheidenheid aan enzymen te producren die deze polysacchariden kunnen afbreken. Daardoor zijn deze schimmels in staat deze polysacchariden af te breken tot kleine monomere suikers die opgenomen kunnen worden door de cel om dan als koolstofbron te dienen. Het in dit proefschrift beschreven onderzoek heeft geleid tot een model voor de wijze waarop *A. niger*, en wellicht ook andere schimmels, deze complexe structuren afbreken. De genen *xlnB* en *xlnD*, die respectievelijk coderen voor endoxylanase B en β-xylosidase, worden als eerste in geringe mate tot expressie gebracht wanneer de schimmel een suikertekort heeft. Deze twee enzymen zorgen ervoor dat kleine hoeveelheden D-xylose worden afgesplitst van het aanwezige xylan. D-xylose wordt vervolgens opgenomen door de schimmel en zorgt ervoor dat via XlnR alle xylanolytische genen sterk geïnduceerd worden. Naast genen coderend voor enzymen die betrokken zijn bij de afbraak van de xylan hoofdketen staan ook verschillende genen die coderen voor cellulolytische enzymen en genen coderend voor enzymen die zijgroepen van de xylan hoofdketen verwijderen onder controle van XlnR. Hierdoor komen naast D-xylose ook andere monosacchariden vrij die op hun beurt andere specifieke polysaccharide-afbrekende enzymsystemen induceren. In dit model is voor XlnR een sleutelrol weggelegd in de regulatie van de afbraak van plantencelwanden. Volgens dit model kunnen alle plantencelwand-afbrekende enzymen geïnduceerd worden in een cascade die de schimmel in staat stelt op een snelle en efficiënte manier in te spelen op zijn omgeving.

Curriculum Vitae

Markus Matheus Catharina Gielkens werd geboren op 14 maart 1969 te ‘s-Hertogenbosch. In 1987 behaalde hij het eindexamen Atheneum-B aan het St. Janslyceum te ‘s-Hertogenbosch. In datzelfde jaar begon hij met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. In 1989 slaagde hij voor het propaedeutisch examen en zette hij de studie voort in de chemisch-biologische oriëntatie. In september 1993 werd het ingenieursdiploma behaald, met de afstudeervakken Moleculaire Genetica van Industriële Micro-organismen (Dr. J. Visser en Dr. M. Kusters-van Someren) en Moleculaire Virologie (Dr. R. Goldbach en Ir. F. van Poelwijk) aan de Landbouwuniversiteit Wageningen, en een stage Moleculaire Genetica bij de Royal Post-graduate Medical School, Londen, Verenigd Koninkrijk (Dr. H. Arst Jr. en Dr. T. Langdon). Van januari 1994 tot januari 1998 was hij als assistent in opleiding (AIO) verbonden aan de sectie Moleculaire Genetica van Industriële Micro-organismen van de Landbouwuniversiteit Wageningen, waar hij onder leiding van Dr. J. Visser en Dr. L. de Graaff het in dit proefschrift beschreven onderzoek uitvoerde. Tevens heeft hij in deze periode met succes afgerond de opleiding Stralingshygiëne deskundigheidsniveau 3 aan de Katholieke Universiteit van Nijmegen. Sinds november 1998 is hij werkzaam als post-doc bij de leerstoelgroep Moleculaire Fythopathologie op het EU project “The role of ABC transporters in pathogenicity and sexual reproduction of *Mycosphaerella graminicola*, the causal agent of septoria tritici leaf-blotch of wheat”.

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Nawoord

Hier ligt het dan eindelijk: …mijn proefschrift. Het beschrijft het onderzoek dat ik uitgevoerd heb bij de sectie Moleculaire Genetica van Industriële Microorganismen. Dat je zo'n boekje niet alleen schrijft blijkt wel uit de namen van de co-auteurs die hebben meegewerkt aan de diverse hoofdstukken. Maar er zijn zeker zoveel mensen die nergens vernoemd staan maar die zeker wel hebben bijgedragen aan het uiteindelijke resultaat.

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Marco