Function and mode of regulation of the transcriptional activator XlnR from *Aspergillus*.

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CHAPTER 1

General Introduction

General introduction

Aspergillus niger

The Kingdom of Fungi includes many different organisms of both ecological and economic importance. Some fungi, called yeast, remain unicellular. However, most fungi are filamentous and exhibit extensive branching. Fungi can be parasitic or symbiotic but most are saprophytic, which means they grow on dead organic matter originating from plants. Filamentous fungi reproduce by forming spores that germinate into filaments called hyphae. The reproductive stage is the mycelium, which is a mass of these hyphal cells. In fungi as the mushroom, a basidiocarp (fruiting body) will develop from the mycelium, in other fungi the mycelium is loosely arranged into a cotton-like structure. An example of the latter is the genus of *Aspergillus*.

In 1792, the priest Micheli was the first to distinguish the stalks and spore heads of *Aspergillus*. He named the fungus *Aspergillus*, since the spore chains radiated from the central structure similar to an aspergillum, which is holy water sprinkler. The genus *Aspergillus* includes more than 600 species. Most of them are saprophytic and only a few are capable of being pathogenic to humans and plants. *Aspergilli* are widespread in the environment; they occur in soil, on plants, in the air, on foods and decaying matter. Several species of the *Aspergillus* genus are studied because of their beneficial characteristics. For example, *Aspergillus nidulans* is, analogous to the yeast *Saccharomyces cerevisiae*, an easily manipulated organism and is used for understanding basic eukaryotic biology and development (Pontecorvo *et al*., 1953). Its complete genome is sequenced and a large number of EST sequences are available to the public (http://aspergillus-genomics.org/asper.fls/asper_blast_dbs.html). Other *Aspergillus* species are used in food, feed or other industries, since they are able to produce organic acids or secrete high levels of enzymes. An *Aspergillus* species well known for its use in the production of soy sauce and alcohols is *Aspergillus oryzae* (Yamane *et al*., 2002). Another species that is used in food industry is *Aspergillus niger*, the organism of interest in this thesis (Fig. 1).

A. niger is used for the production of organic acids, but also for the production of a variety of commercial enzymes. Enzyme preparations derived from *A. niger* find wide application in different industries, including agriculture, textile, detergent, pulp and paper, and food/feed and drink industries (Farrell & Skerker, 1992, Grassin & Fauquembergue, 1996, Maat *et al*., 1992, Nissen *et al*., 1992, Duarte *et al*., 1999). To illustrate the diversity, the enzymes that are used in animal feeds are listed in table 1.

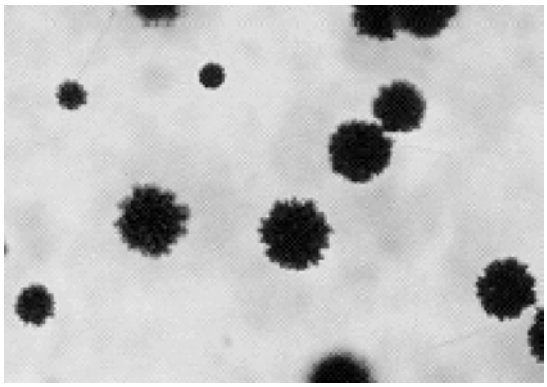

Fig. 1: Picture of *Aspergillus niger*.

Table 1: List of enzymes [a)] produced by *A.* niger used in animal feeds

Enzyme	Function	Current Supported Use
Carbohydrases		
α-Amylase	hydrolyses starch	increases digestibility of starch in pig and poultry diets
Cellulase	breaks down cellulose	increases digestibility of fiber in ruminant and non-ruminant diets
α-Galactosidase	hydrolyses oligosaccharides	increases digestibility of residual oligosaccharides and disaccharides in pig diets
β-Glucanase	hydrolysis of β-glucans, a type of nonstarch polysaccharides (NSP)	reduction of digest viscosity in poultry diets; decreases anti-nutritional effects of NSP; reduces soluble NSP in
β-Glucosidase	hydrolyses cellulose degradation products to glucose	
Glucoamylase	hydrolyses starch with production of glucose	more complete digestion of starch in immature animals
Hemicellulase	breaks down hemicellulose	reduction of digesta viscosity; decrease nutritional effects of NSP; reduces soluble NSP in digesta; increases
Invertase	hydrolyses sucrose to glucose and fructose	
Lactase	hydrolyses lactose to glucose and galactose	
β-Mannanase	hydrolyses β -mannans, a component of hemicellulose	reduction of anti-nutritive β-mannans; reduction of digesta viscosity; increases digestibility of mannans
Pectinase	breaks down pectin	more complete hydrolysis (digestion) of pectins in wheat and corn in piglet diets
Xylanase	hydrolyses xylans	reduction of digesta viscosity; decreases anti-nutritional effects of NSP; reduces soluble NSP in digesta
Lipases		
Lipase	hydrolyses triglycerides	
Proteases		
Protease (general)	hydrolyses proteins	hydrolysis of proteins in feed ingredients in pig and poultry diets
Oxidoreductases		
Catalase	produces water and oxygen from hydrogen peroxide	
Glucose Oxidase	degrades glucose to hydrogen peroxide and gluconic acid	
Phosphatases		
Phytase	hydrolyses phytate	increases digestibility of phytin-bound phosphorus in swine and poultry diets

a) Source Enzyme Technical Association

The plant cell wall

Plant cell walls fulfil an important role in the global carbon cycle; they are the storage site for carbohydrates that serve as a nutrient for many different organisms. The wall of plant cells is composed of approximately 30% cellulose, 30% hemicellulose, and 35% pectin, with 1–5% structural proteins. Variation from these values may be found, particularly in the grasses (Carpita 1996), where, for example, the walls of maize coleoptiles (tissue mantle surrounding the shoot) consist of 55% hemicellulose, 25% cellulose, and only 10% pectin.

A model of a flowering plant cell wall according to Carpita and Gibeaut (1993) is given in Figure 2. Due to its structure, cellulose plays a major role in determining the strength and structural form of the cell wall. Cellulose is a tightly packed microfibril of linear chains of (1->4)β-linked D-glucose. These microfibrils are of indeterminate length and vary in width and in degree of order, depending on source. Hemicelluloses are a heterogeneous group of noncrystalline glycans that are tightly bound in the wall. Dependent on the type of plant cell wall, they mainly consist of xyloglucans or glucuronarabinoxylans. Xyloglucans are linear chains of (1->4)β-D-glucans that hold xylosyl units at the O-6 position of the glycosyl units of the chain. Because xyloglucans are longer than the spacing between cellulose microfibrils, they have the potential to link these microfibrils. Glucuronarabinoxylans are linear chains of (1->4)β-D-xylose with single L-arabinose units at the O-3 and single glucosyluronic acid units at the O-2 of the xylosyl units of the chain. Other polymers that may be found in the hemicellulose fraction of the wall are arabinan, galactan, gluco- and galactoglucomannans, galactomannans, (1->3)β-D-glucans, and arabinoxylans. Some of the hemicelluloses have aromatic side-groups such as ferulate, *p*-coumarate and hydroxycinnemates. Glucuronarabinoxylans can be cross-linked in walls by both esterified and etherified hydroxycinnemates. Ferulate and *p*-coumarate esters can be attached to the O-5 of the arabinosyl units (Harris and Hartley, 1980). The hemicelluloses are not organised into crystalline arrays like cellulose, nor are they randomly arranged in the wall, but they rather appear to lie in a preferred orientation parallel to the cellulose microfibrils (Morikawa *et al*., 1978, McCann *et al*., 1992). This is consistent with the results of binding studies showing that xyloglucan can bind tightly to the surface of the cellulose microfibril (Hayashi, 1989).

Pectins form a gel phase in which the cellulose-hemicellulose network is embedded (Cosgrove, 1997). Like the hemicelluloses, pectins also are a heterogeneous group of polysaccharides, characteristically containing acidic sugars such as glucuronic acid and galacturonic acid. Some pectins have a relatively simple primary structure such as homogalacturonan, a linear polymer of (1-> 4)β-galacturonic acid, with occasional rhamnosyl

residues that put a kink in the chain. Rhamnogalacturonan I (RG I) has repeating subunits of (1->2) α-L-rhamnosyl-(1->2)α-D-galacturonyl disaccharides, with long side chains of arabinans and arabinogalactans.

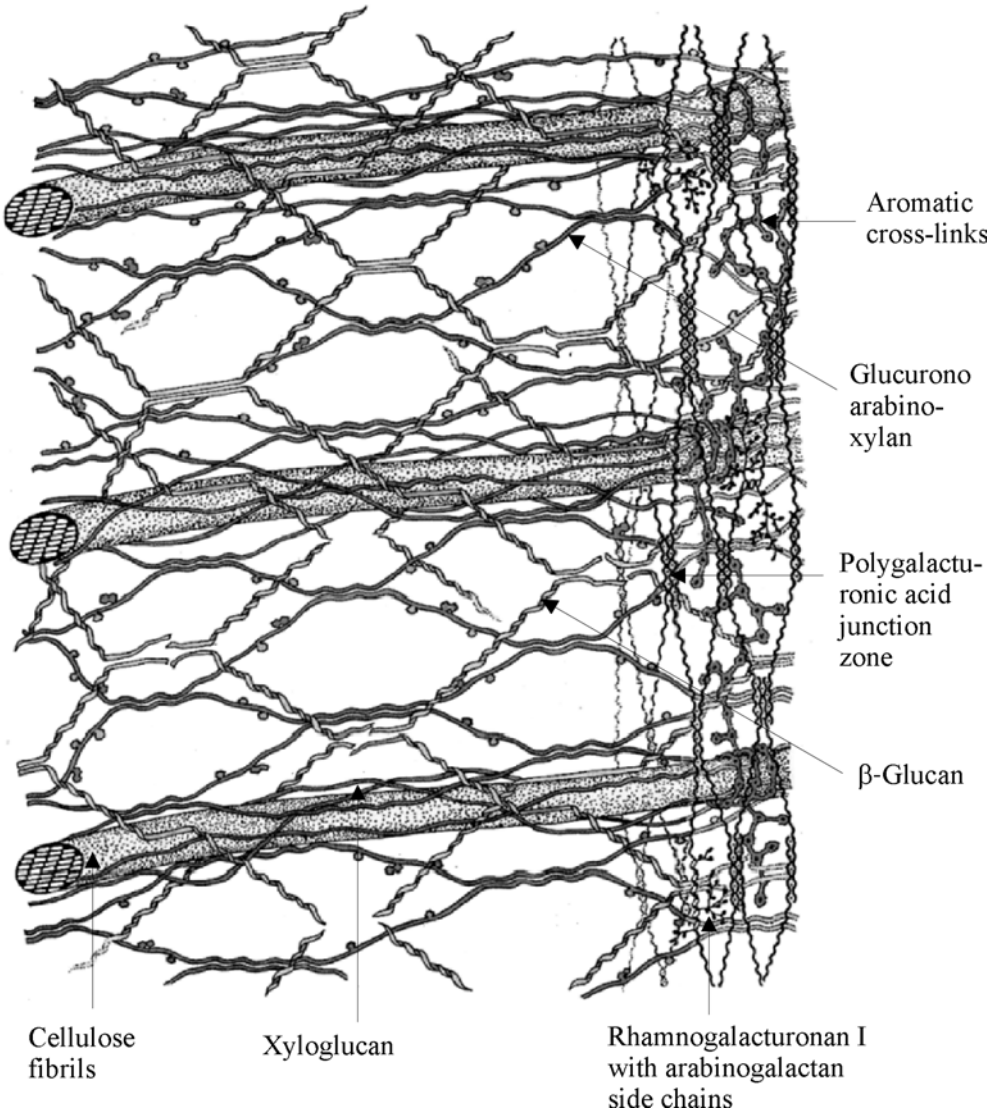

Fig. 2. Plant cell wall (typeII). Modified from Figure 10, Carpita and Gibeaut, 1993)

Enzymatic degradation of plant cell wall polysaccharides.

Saprophytes, which can be both bacteria and fungi such as *A. niger*, grow on dead plant material. They consume the polysaccharides from plant cell walls after degradation. Due to the complex nature of this polysaccharide structure, a variety of enzymes with different functions is required. For example, to degrade the cellulose microfibrils the organism needs a combination of cellulolytic enzymes able to hydrolyse the cellulose in its solid and more soluble state. For the degradation of the different types of hemicelluloses and pectins it needs an even more diverse range of hydrolytic enzymes. The following subparagraphs describe the variety of enzymes involved in degrading the polysaccharides mentioned above.

Cellulose degradation

There are several types of cellulases that cleave the (1->4)β-glycosidic bonds in the cellulose chains. These cellulases are classified as cellobiohydrolases (EC 3.2.1.91) which act as exo-enzymes and release cellobiose, as endoglucanases (EC 3.2.1.4) that attack the cellulose chains by endo-action and as β-glucosidases (EC 3.2.1.21), which hydrolyse cello-oligosaccharides and cellobiose into D-glucose. Some cellulose-degrading enzymes can be equipped with a cellulose-binding domain (CBD) that binds to solid cellulose (Tomme *et al*., 1995, Linder and Teeri, 1997). The CBD is a well-conserved domain, which is connected *via* a linkerpeptide to either the N- or the C-terminus of the catalytic domain of the enzyme. Based on the structure and chemical reaction mechanism of the catalytic domain, the cellulases have been grouped into several families (Henrissat and Bairoch, 1996).

The soft rot fungus *Trichoderma reesei* is one of the best-studied cellulolytic organisms. In *T. reesei* eight genes encoding cellulolytic enzymes have been cloned, two genes encoding cellobiohydrolases, *cbh1*, and *cbh2*, five genes encoding endoglucanases, *egl1*, *egl2*, *egl3*, *egl4* and *egl5*, and one gene encoding a β-glucosidase, *bgl1* (Ilmén *et al*., 1997). All cellulolytic enzymes from *T. reesei* contain a CBD, only for Egl2 it has been shown that in a later stage of cellulose degradation, when the substrate has been solubilised, the CBD is proteolytically removed (Saloheimo *et al*., 1988). Removal of the CBD will give the enzyme different properties so that it will be able to degrade soluble cellulose more efficiently. In *A. niger*, five genes encoding cellulolytic enzymes have been cloned (Table 2, Fig. 3A), two genes encoding endoglucanases, *eglA* and *eglB* (van Peij *et al*., 1998a) (Fig. 3A, 3), one gene encoding β-glucosidase, *blgA* (Fig. 3A, 1), and two genes encoding cellobiohydrolases, *cbhA* and *cbhB* (Fig. 3A, 2). The cellobiohydrolase CbhB harbors a C-terminal CBD, while CbhA, EglA and EglB only consist of a catalytic domain (Gielkens *et al*., 1999).

Hemicellulose degradation

Since xylan is the most common hemicellulose present in the plant cell wall, xylanases are next to cellulases a major group of polysaccharide degrading enzymes. Two classes of enzymes degrade the backbone of xylan: endoxylanases (EC 3.2.1.8) that cleave the xylan backbone into smaller oligosaccharides, which are then further degraded to xylose by β-xylosidases (EC 3.2.1.37). Enzymes that are able to hydrolyse potential side-groups from the xylan main chain are 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 (Biely, 1985). Another modifying enzyme is acetylxylan esterase (EC 3.1.1.72).

Several different xylanases have been identified in *Aspergillus* species. In *A. niger*, two genes encoding endoxylanases have been cloned, *xlnB* (Kinoshita *et al*., 1994) and *xlnC* (Gielkens, unpublished) (Fig. 3B, 5), and one β-xylosidase, *xlnD* (van Peij *et al*., 1997) (Fig. 3B, 6). In addition, several genes encoding accessory enzymes have been cloned (Table 2); an α-L-arabinofuranosidase gene *abfB* (Fig. 3B, 1) (Flipphi *et al*., 1993), an arabinoxylan arabinofuranohydrolase gene *axhA* (Fig. 3B, 1) (Gielkens *et al*., 1997) an acetylxylan esterase gene *axeA* (Fig. 3B, 3) (de Graaff *et al*., 1992), an α-glucuronidase gene *aguA* (Fig. 3B, 2) (de Vries *et al*., 1998), and a feruloyl esterase gene *faeA* (Fig. 3B, 4) (de Vries *et al*., 1997). See also 'Transcription regulation of the xylanolytic enzyme system of *Aspergillus*' (van Peij, 1999, Ph.D. thesis Wageningen Agricultural University) and 'Accessory enzymes from *Aspergillus* involved in xylan and pectin degradation' (de Vries, 1999, Ph.D. thesis Wageningen Agricultural University). From *Aspergillus tubingensis* a third gene encoding an endoxylanase, *xlnA*, that is not present in *A. niger*, was cloned (de Graaff *et al*., 1994). Other enzymes involved in hemicellulose degradation are galactosidases, xyloglucanases and endoarabinases.

Pectinases

Pectinases can be classified, based on their substrate specificity, into a group of homogalacturonan and a group of rhamnogalacturonan specific enzymes. The pectin backbone can be hydrolysed by pectin lyases, pectate lyases and polygalacturonases. Also several accessory enzymes that are active towards the side chains of pectin are necessary to fully degrade the polysaccharide. From *A. niger* several enzymes that are active on the homogalacturonan part of pectin were identified; pectin methyl- and acetyl-esterase (EC 3.1.1.11 and EC 3.1.1.6), endopolygalacturonase (EC.3.2.1.15), exopolygalacturonase (EC 3.2.1.67), pectate lyase (EC 4.2.2.2) and pectin lyase (EC 4.2.2.10). This has been described in 'Pectinases

of *Aspergillus*: A molecular and biochemical characterisation' (Pařenicová, 2000, Ph.D. thesis Wageningen University).

Table 2: Enzymes from *A. niger* involved in cellulose and arabinoxylan degradation. The position of action of the enzymes is pointed out in Fig. 2.

Protein	Enzyme	Mode of action
BlgA	β-Glucosidase A	1 (Fig. 3A)
CbhA	Cellobiohydrolase A	2 (Fig. 3A)
CbhB	Cellobiohydrolase B	2 (Fig. 3A)
EglA	Endoglucanase A	3 (Fig. 3A)
EglB	Endoglucanase B	3 (Fig. 3A)
AbfB	α-L-Arabinofuranosidase B	1 (Fig. 3B)
AguA	α-Glucuronidase A	2 (Fig. 3B)
AxeA	Acetylxylan esterase	3 (Fig. 3B)
AxhA	Arabinoxylan hydrolase A	1 (Fig. 3B)
FaeA	Feruloyl esterase A	4 (Fig. 3B)
XlnB	Endoxylanase B	5 (Fig. 3B)
XlnC	Endoxylanase C	5 (Fig. 3B)
XlnD	β-Xylosidase	6 (Fig. 3B)

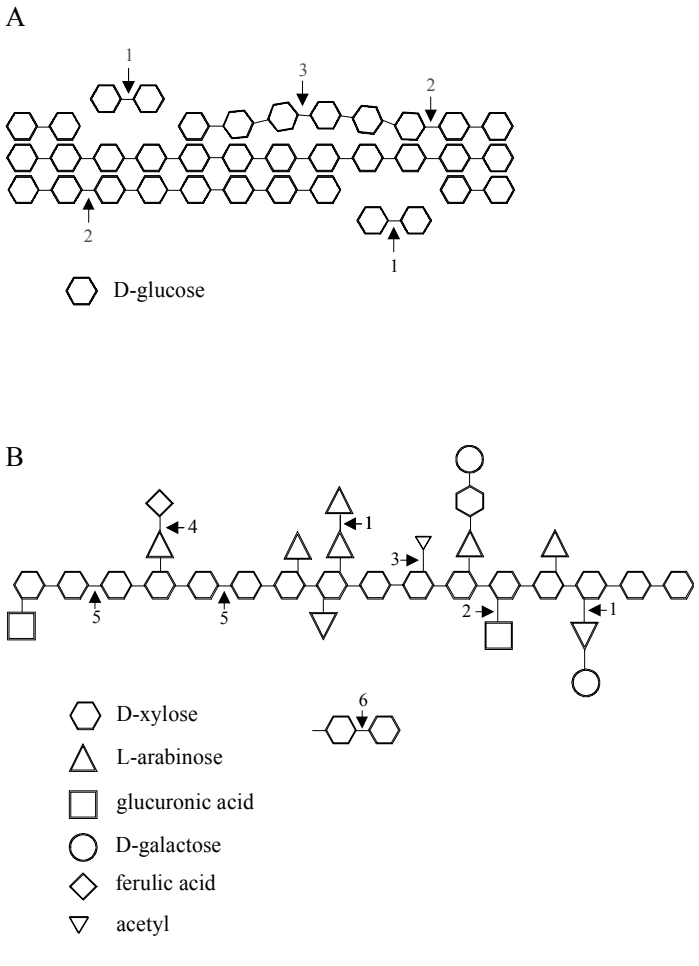

Fig. 3. Hypothetical cellulose (A) and xylan (B) structure and mode of action of the polysaccharide degrading enzymes identified in *A. niger* (see Table 2).

Regulation of cellulase and xylanase expression.

Which type of polysaccharide degrading enzymes an organism requires, depends on the carbon source that is available. To utilise its energy in an efficient way, the organism regulates its carbon metabolism. In general this occurs by controlling the expression of polysaccharide degrading enzymes by induction *via* a specific transcriptional activator and by carbon catabolite repression. Carbon catabolite repression is a global regulatory mechanism that represses the expression of genes involved in utilisation of alternative carbon sources in the presence of energetically more favourable ones such as D-glucose and D-fructose.

The expression and secretion of polysaccharide degrading enzymes starts with the uptake of signal molecules that act, directly or indirectly, as inducer. It is hypothesised that these signal molecules, whose nature depends on the polymer present, are generated through the action of enzymes that are produced and secreted by the cell at low constitutive levels (van Peij *et al*., 1999, Gielkens *et al*., 1999). The signal molecule is taken up and activates a specific induction pathway that results in the synthesis of the certain enzymes required to degrade the polymer. A transcriptional activator that controls the transcription of the structural genes encoding the polysaccharide degrading enzymes is part of the induction pathway.

Pathway-specific induction.

In the presence of a carbon source like D-galactose, D-maltose, or polysaccharides such as cellulose or xylan, a set of pathway specific enzymes is produced. As mentioned before, in most cases this process is controlled at the level of gene expression by a transcriptional activator. In fungi, many metabolic pathways are regulated by members of the $Zn(II)_2Cys_6$ binuclear cluster family of transcriptional activators, of which GAL4 from *Saccharomyces cerevisiae* is the prototype. GAL4 controls the synthesis of D-galactose-metabolising enzymes (Johnston and Hopper, 1982, Giginger *et al*., 1985). It recognises and binds to specific upstream activating sequences (UAS) of the *gal* structural genes. Activation of GAL4 in the presence of D-galactose requires the interaction of an inhibitory protein GAL80 and a signal transducer Gal3 (Zenke *et al*., 1996, Suzuki-Fujimoto *et al*., 1996). Another Zn-finger transcription activator from *S. cerevisiae* that is involved in carbon metabolism is MAL63, which regulates the maltose-inducible expression of the *mal* structural genes encoding maltose permease and D-maltase (Needleman, 1991). The structural genes, *mal61* and *mal62*, are co-ordinately and divergently transcribed from a common promoter (Levine *et al*., 1992).

Besides the various types of sugar molecules, fungi can utilise ethanol as a sole carbon source *via* its conversion into acetaldehyde and acetate. In *Aspergillus nidulans*, the ethanol utilisation pathway is a well-studied example of a transcriptionally controlled catabolic system.

Induction of ethanol catabolism requires the action of the transcriptional activator AlcR (Felenbok *et al*., 1988). AlcR activates both the transcription of the structural genes *alcA* and *aldA* and its own transcription (Lockington *et al*., 1987), which enables a quick adaptation towards ethanol conversion and amplifies the inducing signal. Another carbon source that can be utilised by *A. nidulans* is proline. The genes encoding enzymes involved in this process, *prnB*, *prnC*, *prnD*, and *prnX* are under control of the transcription factor PrnA (Cazelle *et al*., 1998). Since proline is nitrogen and carbon source in *A. nidulans*, the pathway is subject to both nitrogen repression and carbon metabolite repression (Arst and Cove, 1973, Bailey and Arst 1975). In this aspect it differs from the *S. cerevisiae* proline utilisation pathway, which is regulated by the PrnA isofunctional protein PUT3 and only subject to nitrogen metabolite repression (Xu *et al*., 1995).

The subject of study in this thesis is the pathway specific transcriptional activator XlnR from *A. niger* (van Peij *et al*., 1998a). XlnR was first identified as a xylanase activity regulator (van Peij *et al*., 1998a) but was later shown to also regulate other hemicellulases (van Peij *et al*., 1998b) and cellulases (Gielkens *et al*., 1999). Until now 11 genes, encoding cellulolytic and hemicellulolytic enzymes, were shown to be regulated by XlnR (van Peij *et al*., 1998b, Gielkens *et al*., 1999). The XlnR-binding site, which could be found in the promoter of all XlnR-regulated genes, was determined to be 5‘-GGCTAA-3‘. In *T. reesei*, recently two genes encoding cellulase regulators were identified, *ace1* and *ace2* (Saloheimo *et al*., 2000, Aro *et al*., 2001 Aro *et al*., 2003). Ace1 is a repressor of cellulase and xylanase expression and recognises 5’-AGGCAAA-3’ and some 5’-AGGCA-3’ sites in the *cbh1* promoter. Ace2 is an activator binding to the sequence 5’-GGCTAATAA-3’. In that respect, Ace2 appears to be similar to XlnR from *A. niger* although there is no sequence similarity between the two proteins.

Carbon catabolite repression.

In the presence of D-glucose or other readily metabolisable sugars, the synthesis of enzymes that degrade alternative carbon sources is repressed *via* carbon catabolite repression. In this energy saving mechanism a repressor protein inhibits the transcription of many target genes by binding to specific sequences in the promoters of these genes.

In *A. nidulans*, this repressor protein is encoded by the gene *creA* that was cloned and characterised by Dowzer and Kelly (1989, 1991). CreA represses genes encoding enzymes involved in the degradation of pectin, cellulose, xylan, ethanol and proline (Ruijter and Visser, 1997). In case of the ethanol regulon it has been shown that CreA operates at two levels. It represses both the transcription of the *alcR* gene, the transcriptional activator for the *alc* genes, and the structural gene *alcA*, encoding alcohol dehydrogenase I (Kulmburg *et al*., 1993, Mathieu

and Felenbok, 1994). Analysis of the CreA consensus sites in the *alcR* promoter showed that CreA acts by two different mechanisms: by competing with AlcR for the same DNA region and by direct repression that presumably affects the transcriptional machinery (Mathieu *et al*., 2000). In contrast, in case of the proline utilisation cluster, CreA only operates at the level of the structural genes. The expression of *prnA*, encoding the transcriptional activator PrnA that regulates *prnB* and *prnD* (Cubero and Scazzzocchio, 1994), was shown to be constitutive and not regulated by CreA (Cazelle *et al*., 1998). The gene encoding the CreA protein from *A. niger* was also cloned and characterised (Drysdale *et al*., 1993) and has 82% overall identity with the *A. nidulans* CreA protein (Ruijter and Visser, 1997). In *A. niger*, CreA was shown to repress the transcription of genes involved in arabinan (Flipphi *et al*., 1994), xylan and cellulose (de Vries *et al*., 1999) degradation. In *T. reesei*, D-glucose repression is mediated by Cre1 (Strauss *et al*., 1995, Ilmén *et al*., 1996a). Cre1 represses the transcription of a number of genes involved in degradation of hemicelluloses and celluloses (Ilmén *et al*., 1996b, Margolles-Clark, 1997, Ilmén *et al*., 1997). Since the end-product of cellulose degradation is D-glucose, the activity of cellulases will result in feedback repression of the corresponding genes *via* Cre1. A comparable effect was found in *A. niger*; in certain concentrations, the degradation products of less-favourable carbon sources as xylan, have a repressing effect on the pathway specific genes (de Vries *et al*., 1999a). Apparently in these degradation pathways, the balance between induction by the specific regulator and repression by the repressor protein determines the expression levels of the genes encoding the polysaccharide degrading enzymes.

In *S. cerevisiae* D-glucose repression is mediated by MIG1 (Nehlin and Ronne, 1990), which represses the transcription of genes encoding activators such as GAL4 (Laughon and Gesteland, 1984) and MAL63 (Needleman, 1991). For MAL63-mediated maltose induction it was reported that there is also D-glucose inhibition through a MIG1-independent mechanism. This D-glucose inhibition mechanism requires SNF1 protein kinase for the synthesis of maltose permease, whose function is essential for maltose induction (Hu *et al*., 2000). Genes similar to *mig1* have been cloned in the other yeasts such as *Kluyveromyces lactis* (Cassart *et al*., 1995) and *Schizosaccharomyces pombe* (Tanaka *et al*., 1998).

Domain organisation and regulation of transcription factors

In fungi, metabolic pathways are in many cases regulated by members of the zinc binuclear cluster family of transcription factors. Except for a few similarities such as an N-terminal DNA-binding site and a C-terminal activation domain, most members of this family have unique characteristic features. They differ in size, domain organisation, and mechanism of regulation.

They are regulated at transcriptional, post-transcriptional or post-translational level or a combination of these.

Most of the information about structure-function relations and regulatory mechanisms of Zn-finger activators is obtained from *S. cerevisiae*. A number of studies were done on Zn-finger activators from filamentous fungi such as *Aspergillus* and *Trichoderma*. The domain organisation and mechanism of regulation of several Zn_2Cys_6 binuclear cluster transcription factors are discussed below.

Zinc finger activators from *S. cerevisiae*

GAL4

The *Gal* gene regulator GAL4 (881 amino acids) from *S. cerevisiae* is the best-characterised member of the Zn_2Cys_6 binuclear cluster family of transcription factors. Besides an N-terminal DNA-binding domain and a C-terminal activation domain (amino acids 768 to 881), GAL4 harbours an inhibitory domain in its central region involved in D-glucose repression (Stone and Sadowski, 1993). Activation of GAL4 requires the interaction between an inhibitory protein (GAL80) and a signal transducer protein (GAL3). In the absence of D-galactose, GAL80 inhibits the GAL4 activity by binding to its activation domain. In the presence of D-galactose, GAL3 interacts with GAL80, removing it from GAL4 at the *gal* gene promoter (Blank *et al*., 1997, Peng and Hopper, 2000, Peng and Hopper, 2002). This D-galactose-triggered GAL3-GAL80 association occurs in the cytoplasm and results in activation of GAL4 in the nucleus (Peng and Hopper, 2002). GAL4 becomes phosphorylated on multiple serine residues if it activates transcription. These phosphorylations are mediated by the cyclin-dependent protein kinases of the RNA Pol II holoenzyme (Hirst *et al*., 1999). One phosphorylation at S699 that is mediated by the holoenzyme-associated kinase Srb10, was shown to be a second signal, besides GAL3-galactose, controlling GAL4 activity (Rohde *et al*., 2000).

MAL63

The regulator of maltose fermentation MAL63 consists of 470 amino acids and has a DNA-binding domain in the N-terminal 100 residues. Deletion analysis of MAL63 was used to identify other functional domains. Residues 60-283 form a functional core region including an activation domain and residues 251-299 are required for inhibition of MAL63. The region C-terminal to residue 215 contains a D-maltose-responsive domain that acts to relieve the inhibitory effect on the activation domain. Different from GAL4, regulation of MAL63 does not involve a GAL80-like negative repressor protein (Hu *et al*., 1999). Since amino acid mutations that cause constitutivity in MAL63 can be suppressed by intragenic mutations, it was suggested

that MAL63 is regulated through specific intramolecular protein-protein interactions (Danzi *et al*., 2000).

LEU3

The regulator of the leucine biosynthetic pathway, LEU3, consists of 886 amino acids, exists as a dimer both in the presence and absence of DNA and occurs in a phosphorylated and non-phosphorylated form (Sze and Kohlhaw, 1993). The activation domain of LEU3 is located within the C-terminal 30 residues and its middle region is essential for the modulation process. LEU3 undergoes intramolecular changes mediated by α-IMP, an intermediate in the pathway. In the presence of α-IMP, there is unmasking of the activation domain and subsequent activation of transcription. In the absence of α-IMP LEU3 represses transcription. A mutant in which two aspartates in the activation domain, D872 and D874, were changed into aspagarines, was permanently masked. Intragenic suppressors of this mutant yielded mutations in three very short regions between positions 604 and 741 in the C-terminal half of the middle region. These suppressor mutations caused constitutivity, which is consistent with an involvement of the residues in masking (Wang *et al*., 1997, Wang *et al*., 1999, Kohlhaw, 2003).

PUT3

Like most of the members of the Zn2Cys6 binuclear cluster family, the transcriptional activator PUT3 binds to the promoter of its target genes in the presence and absence of proline (Axelrod *et al*., 1991). PUT3 responds to two signals, the presence of proline and the absence of a preferred nitrogen source. Hyperphosphorylation of PUT3 is correlated with growth on non-preferred nitrogen sources and maximum transcription of the PUT3 target genes is achieved when proline serves as the sole carbon source (Huang and Brandiss, 2000). As constitutive mutants in PUT3 are all dominant, it was suggested that the activity of PUT3 is controlled by changes in its conformation (Marczak and Brandiss, 1989). The 'on' conformation in the presence of proline can be mimicked by disruption of its 'off' conformation for instance by specific amino acid changes (des Etages *et al*., 2001).

Zinc finger transcription factors from filamentous fungi

PrnA from *A. nidulans*

The transcriptional activator PrnA from *A. nidulans*, which is isofunctional to PUT3 from *S. cerevisiae*, is 818 amino acids long and harbours a tripartite nuclear localisation signal in its N-terminus. PrnA contains a putative dimerisation domain, 54 residues downstream from the last cysteine of the Zn cluster. This dimerisation domain is followed by an acidic region from residues 120 to 152, predicted to form an α-helix. A second potential coiled-coil region is located in the C-terminal part of the protein between residues 600 and 650. In the C-terminus,

there is a glutamine-rich region (residues 701 to 718), which could be an activation domain (Cazelle *et al*., 1998). The transcription of *prnA* is neither self-regulated nor affected by carbon and/or nitrogen repression. Proline induction is essential for *in vivo* binding of PrnA to the DNA, but not for its nuclear entry (Pokorska *et al.,* 2000, Gomez, *et al.,* 2002). Thus, PrnA is constitutively present in the nucleus, but only bound to the DNA in response to the presence of proline. Its mode of regulation when bound to the DNA still remains unclear.

AlcR from *A. nidulans*

The AlcR transcription regulator is, as PrnA, translocated into the nucleus in the absence of inducer. The signal for nuclear entrance or/and retention resides within the N-terminal region, which plays a dual role since it also serves as a DNA recognition unit. Although the DNA-binding motif and its mode of binding were examined extensively (Cahuzac *et al*., 2001), little is known about structure-function organisation of other domains of AlcR from *A. nidulans*. Deletion of several regions of AlcR suggested that the integrity of the complete coding structure, which consists of 821 amino acids, is essential for its activity. Although in other Zn2Cys6 proteins the main activation domain is C-terminally located, in AlcR a putative activation domain is localised immediately C-terminal to the zinc-cluster, between residues 60 and 197 (Felenbok *et al.,* 2001). In GAL4 a second weak activation domain is also localised close to the zinc finger motif (Ma and Ptashne, 1987).

CreA from *A. nidulans*

The glucose repressor CreA from *A. nidulans* was shown to be regulated at transcriptional as well as at post-transcriptional level. On a transcriptional level *creA* is regulated in a complex way, mRNA levels differ dependent on whether there is D-glucose depletion or a repressing or derepressing carbon source present. Formation of a CreA-DNA complex upon transfer from derepressing to repressing conditions indicates post-transcriptional regulation since this process requires *de novo* protein synthesis (Strauss *et al.,* 1999).

XlnR from *A. niger*

XlnR from *A. niger* is also a typical zinc binuclear cluster protein consisting of 875 amino acids. Although most zinc binuclear cluster proteins bind as a dimer, it seems that XlnR binds as a monomer. The XlnR-binding sites, 5'-GGCTAA-3', do not occur in a palindrome and varies in both orientation and in number (van Peij *et al.,* 1998a). AlcR is also thought to act as a monomer (Lenouvel *et al.*, 1997). Computer analysis of the amino acid sequence of XlnR predicted two putative coiled-coiled domains as in PrnA, one directly C-terminal to the DNA-binding domain and one at the C-terminal part of XlnR. A single amino acid substitution in this second coiled-coil domain resulted in a *xlnR* loss-of-function mutant, which indicates that this region is

important for XlnR function. Two other *xlnR* loss-of-function mutations were found in the C-terminal region, one of them 12 amino acids and the other one 52 amino acids from the C-terminal end. This part of the protein might be involved in XlnR activity as in other Zn2Cys6 proteins, such as GAL4, LEU3 and PUT3 from *S. cerevisiae* (Hoy *et al*., 1993, des Etages *et al*., 1996, Wang *et al*., 1997). Transcription activation domains are commonly rich in glutamine, proline, isoleucine or acidic residues (aspartate and glutamate). In XlnR these amino acids are found in a cluster about 30 residues from the C-terminal end.

The examples given above show that regulation of Zn binuclear cluster transcription activators can take place in different ways. In general, they are regulated at the level of changes in their activity while bound to DNA. These changes in activity can be achieved by inter- or intramolecular protein-protein interaction; by repressor binding, *via* changes in conformation, unmasking of activation domains or complex formation.

Aim and outline of this thesis

In preceding research the gene encoding the transcription factor XlnR from *A. niger* was cloned based on its property to regulate genes encoding extracellular xylanolytic enzymes. Many fungi are capable of degrading plant cell wall polysaccharides; XlnR was the first gene that was isolated encoding a transcription factor of a plant cell wall degrading enzyme system. The XlnR protein harbours a zinc binuclear cluster domain with similarity to the zinc clusters of the GAL4 superfamily of transcription factors. Except for the DNA-binding domain, no sequence similarity of XlnR with other zinc binuclear cluster proteins was found. Several putative structural/functional domains in XlnR were identified, however no information about the molecular mechanisms that underlie the XlnR regulatory pathway was available at the start of this Ph.D. research.

The aim of the research described in this thesis was to investigate the role of XlnR in plant cell wall degradation by *A. niger* in more detail. The spectrum of genes of which the transcription is regulated by XlnR was investigated. For this an *A. niger* differential cDNA library was constructed by subtracting the cDNA of a D-xylose induced *xlnR* loss-of-function mutant from the cDNA of a D-xylose induced wild-type and a *xlnR* multiple copy strain. The resulting cDNA libraries, enriched in fragments of genes controlled by XlnR, were screened for new genes encoding enzymes involved in plant cell wall degradation (Chapters Two and Three).

Since many fungi produce polysaccharide-degrading enzymes, the presence of a regulatory pathway as in *A. niger* might be widespread. Also in *A. nidulans*, the transcription of

cellulolytic and xylanolytic genes was shown to be controlled by a XlnR orthologue (Chapter Four).

Another objective of this research was to obtain a better understanding of the molecular mechanisms that are involved in the expression of the XlnR-controlled enzyme system. For this an *A. niger* regulatory mutant constitutive for xylanase activity was isolated and characterised. The role of D-glucose repression in XlnR-controlled regulation of transcription was examined using a CreA derepressed *A. niger* mutant. Analysing transcript levels of several XlnR-controlled genes resulted in a revised model for the regulation of the xylanolytic system (Chapter Five).

Also the XlnR protein itself was investigated, with the aim to unravel structure-function relations. This was done by constructing several XlnR partial deletion-mutants and mutants with single amino acid substitutions. Xylanase activity assays and Gfp-XlnR fusion's were used to investigate possible post-translational regulatory mechanisms and putative functional domains (Chapter Six).

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CHAPTER 2

The *Aspergillus niger* transcriptional activator XlnR which is involved in the degradation of the polysaccharides xylan and cellulose, also regulates D-xylose reductase gene expression

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Abstract

Screening of an *Aspergillus niger* differential cDNA library, constructed by subtracting cDNA fragments of a *xlnR* loss-of-function mutant from wild-type cDNA fragments, resulted in the cloning of the gene encoding D-xylose reductase (*xyrA*). Northern blot analysis using an *A.niger* wild type strain, a *xlnR* multiple-copy strain and a *xlnR* loss-of-function mutant confirmed that the *xyrA* gene is regulated by XlnR, the transcriptional activator of the xylanolytic enzyme system in *A.niger*. D-xylose reductase catalyses the NADPH-dependent reduction of D-xylose to xylitol, which is the first step in D-xylose catabolism in fungi. Until now, XlnR was shown to control the transcription of genes encoding extracellular hydrolytic enzymes involved in cellulose and xylan degradation. In the present study, we show that *A.niger* is able to harmonize its sugar metabolism and extracellular xylan degradation *via* XlnR by regulating the expression of XyrA.

Introduction

The carbon cycle in nature starts with the fixation of carbon dioxide by photosynthetic plants. Part of the sugars generated is used by the plant to synthesise the structural polysaccharides, which are present in the plant cell wall. These polysaccharides are pectin, cellulose and hemicellulose, the latter being mainly xylan (Carpita and Gibeaut, 1993). Micro-organisms that are able to degrade these plant cell wall polysaccharides play an essential role in the transfer of these sugars to non-photosynthetic organisms. Although the biochemistry of the process of cellulose and xylan degradation has been studied in great detail, the mechanism by which the organism senses the substrate and adapts its carbon metabolism to polysaccharide degradation is still largely unsolved.

Aspergillus niger is a saprophytic fungus able to degrade xylan by the extracellular secretion of a variety of hydrolytic enzymes. Xylan is composed of a main chain of β-1,4-linked D-xylose residues, which can be decorated with side groups such as D-glucuronic acid, L-arabinose, *p*-coumaric acid and ferulic acid. To degrade this polysaccharide, *A. niger* needs to express a variety of genes encoding xylan modifying and degrading enzyme functions. The various side-groups are cleaved by enzymes such as arabinoxylan arabinofuranohydrolase (AxhA, EC 3.1.1.72) (Gielkens *et al*., 1997), which cleaves α-1,2 and α-1,3 linked arabinofuranosyl residues. The enzyme α-glucuronidase A (AguA, E.C.3.2.1.139) (de Vries *et*

al., 1998) cleaves α-1,2 linked glucuronic acid residues and feruoylesterase A (FaeA) (de Vries *et al*., 1997) hydrolyses feruoylester bonds from arabinofuranosyl residues. The backbone of xylan is cleaved by two different endoxylanases (XlnB and XlnC, EC 3.2.1.8) (de Graaff *et al*., 1994) generating D-xylose and xylo-oligosaccharides like xylobiose and xylotriose. These oligosaccharides in turn are cleaved by β-xylosidase (XlnD, EC 3.2.1.37) (van Peij *et al*., 1997) to generate the monosaccharide D-xylose.

In *A.niger,* the expression of the xylanolytic enzyme system is regulated by the transcriptional regulator XlnR (van Peij *et al*., 1998a). XlnR is a zinc binuclear cluster type of protein similar to, for example, the *Saccharomyces cerevisiae* GAL4 (Johnston and Dover, 1987) and the *Aspergillus nidulans* AlcR (Felenbok, 1988) transcription factors. The expression of more than 10 genes encoding extracellular hydrolytic enzymes involved in the degradation of xylan and unexpectedly also of cellulose were shown to be controlled by XlnR (van Peij *et al*., 1998b, Gielkens *et al*., 1999, de Vries *et al*., 1999). The main product of xylan degradation, D-xylose, induces the expression of these genes via XlnR. In fungi, the reduction of D-xylose to xylitol, which is catalysed by D-xylose reductase, is the first step in D-xylose catabolism. Subsequently xylitol is oxidised to D-xylulose, which is phosphorylated to xylulose-5-phosphate and channelled *via* the pentose-phosphate pathway (Fig.1) into glycolysis (Chiang and Knight, 1960).

To further investigate the spectrum of genes controlled by XlnR in *A.niger*, a differential cDNA library was constructed. Screening of this library resulted in the cloning of the gene encoding D-xylose reductase (*xyrA*). Thusfar, it was shown that in *A.niger* the transcription of genes encoding extracellular xylanolytic and cellulolytic enzymes is XlnR regulated. We now show that the transcription of the gene encoding the intracellular D-xylose reductase is regulated by XlnR, this allows the fungus to adapt its sugar metabolism to extracellular xylan degradation.

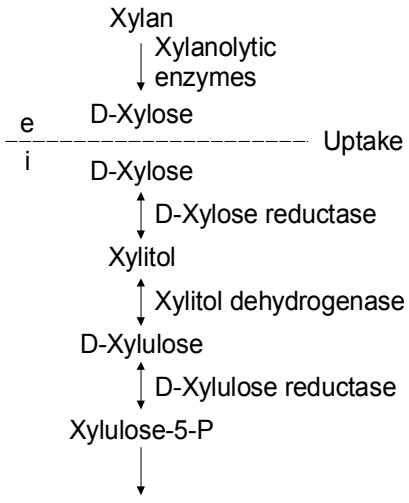

Fig.1. Schematic representation of the first steps in the D-xylose catabolic pathway in *A.niger* (cf. Witteveen *et al*., 1989). The D-xylose is generated extracellularly (e) by the action of the xylanolytic enzymes, is taken up and intracellularly (i) catabolised to xylulose-5-phoshate, which enters the pentose phosphate pathway.

Results

Isolation and characterisation of the *xyrA* gene of *A.niger*

Screening of an *A.niger* differential cDNA library, constructed by subtracting the cDNA of a D-xylose induced *xlnR* loss-of-function mutant from the cDNA of a D-xylose induced wild type strain, resulted in the cloning of *Rsa*I fragments of XlnR regulated genes. One of these *Rsa*I fragments showed a high sequence homology to a gene encoding D-xylose reductase in the yeasts *Pichia stipitis* (Takuma *et al*., 1991) and *Kluyveromyces lactis* (Billard *et al*., 1995). Because this cDNA fragment potentially originates from the D-xylose reductase encoding *xyrA* gene of *A. niger*, the *Rsa*I fragment was used to isolate the complete cDNA from an *A.niger* cDNA library (Gielkens *et al*., 1999), resulting in the plasmid pIM4401. After screening of an *A.niger* genomic library using the same *Rsa*I fragment, a 5.2 kb *Bam*HI fragment was obtained from a positive λ-phage and cloned in pUC19, resulting in the plasmid pIM4400 (Fig. 2A). By comparing the sequence of the cDNA and the genomic DNA, two introns of 62 and 61 bp in size were localised. The open reading frame of the *xyrA* gene was found to be 957 bp, encoding a peptide of 319 amino acids, with a predicted molecular mass of 36 kDa. Analysis of the promoter region of *xyrA* revealed two putative XlnR binding sites (van Peij *et al*., 1998b) located 204 and 312 bp upstream of the putative translation start codon.

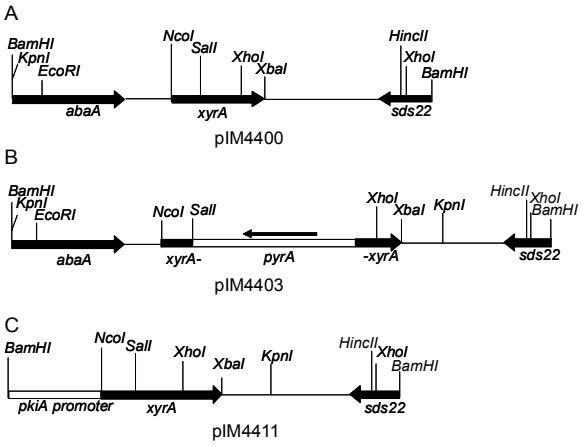

Fig.2. Restriction maps of the plasmids used in this study. All constructs were made in pUC19.
A. Map of *xyrA* genomic region in pIM4400. B. Map of the *xyrA* disruption plasmid pIM4403. C. Restriction map of the *pkiA*-promoter-*xyrA* expression construct pIM4411. By means of site directed mutagenesis, a *NcoI* site was introduced in the translation start of a *pkiA*-promoter and fused to a 3.2 kb *NcoI-KpnI* fragment which contains the *xyrA* gene.

Upstream the *xyrA* gene on this 5.2 kb *Bam*HI fragment, the 3' end of a gene with a high sequence homology to the *abaA* gene of *Aspergillus nidulans* was found. The *abaA* gene (Andrianopoulos *et al*., 1994) encodes a regulator protein involved in *Aspergillus* development. Downstream the *xyrA* gene, a gene highly homologous to the *sds22+* encoding gene of *Schizosaccharomyces pombe* was found. The sds22+ protein is able to enhance a protein phosphatase-1 dependent dephosphorylation activity that is essential in mid-mitose (Ohkura and Yanagida, 1991).

Table 1. *Aspergillus niger* strains

Strains	Genotype	Relevant properties	Reference
N902	*argB15, cspA1, fwnA1, metB10, pyrA5*	Wild type	
N902::230-25.12 (3xlnR9)	*argB15, cspA1, fwnA1, metB10*	10 copies *xlnR*	van Peij *et al*., 1998b
NXA1-4	*argB13, nicA1, pyrA6, xlnR1,*pIM130#6	*xlnR* loss-of-function mutant	van Peij *et al*., 1998a
NW199	*fwnA6, leuA5, goxC17, pyrA6,* Δ*xlnR::*pIM240	*xlnR* disruption mutant	van Peij and de Graaff, unpublished
NW219	*cspA1, nicA1, leuA1, pyrA6*	Wild type	
NW219::4400-3 (MC3)	*cspA1, n icA1, leuA1*	3-4 copies *xyrA*	This work
NW219::4400-12 (MC12)	*cspA1, nicA1, leuA1*	8-10 copies *xyrA*	This work
NW219::4400-19 (MC19)	*cspA1, nicA1, leuA1*	15-20 copies *xyrA*	This work
NW280	*cspA1, nicA1, leuA1* Δ*xyrA:*PYRA+	*xyrA* disruption mutant	This work
NW219::4411-5 (TR5)	*cspA1, nicA1, leuA1*	*xyrA* gene under control of *pkiA* promoter	This work
NW219::4411-12 (TR12)	*cspA1, nicA1, leuA1*	*pkiA* promoter	This work

XlnR controls transcription of *xyrA*

In order to verify the XlnR controlled transcription of *xyrA*, Northern blot analysis was performed. For this, RNA isolated from *A.niger* N902 (wild type), NXA1-4 (*A.niger xlnR* loss-of-function-mutant, van Peij *et al.*, 1998a) and *A.niger xlnR* multiple copy strain 3xlnR9, which harbours 10 copies of the *xlnR* gene (N902::230-25.12, van Peij *et al*., 1998b) (Table 1). All strains were precultured for 18 h using Minimal Medium (MM) with 100 mM D-fructose as a carbon source and were then transferred for 6 h to MM with 50 mM D-xylose as an inducing carbon source. Compared to the wild type, increased levels of *xyrA* transcription were observed in the *xlnR* multiple copy strain, while no transcription was found in the *xlnR* loss-of-function mutant (Fig. 3).

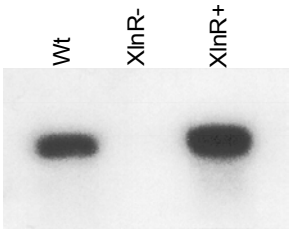

Fig.3. Northern blot analysis of *xyrA* transcription in *A.niger* wild type (wt), *xlnR* loss-of-function mutant (XlnR-) and *xlnR* multiple copy tranformant (XlnR+). The strains were precultured in MM containing 100 mM D-fructose as a carbon source. Total RNA was isolated from mycelium grown for 6 h after transfer to MM containing 50 mM D-xylose as an inducing carbon source.

Functionality of the *xyrA* gene

To confirm the functionality of the *xyrA* gene cloned, *xyrA* multiple-copy strains and a *xyrA* disruption mutant were constructed. *XyrA* multiple-copy strains were obtained by co-transformation of the NW219 wild-type strain using pIM4400 and pGW635 (Goosen *et al*., 1987). The latter plasmid contains the *A.niger pyrA* gene to be able to select for uridine prototrophy. Identification of multiple copy transformants was carried out by measuring the NADPH-dependent D-xylose reductase activity in cell lysates from mycelium of 20 different transformants and of the wild type strain after a 6 h transfer to D-xylose. Three multiple copy transformants were selected which contained, as estimated from the Southern blot, 3-4 (MC3), 8-10 (MC12) and 15-20 (MC19) copies of the *xyrA* gene. These three multiple copy strains had about 4, 10 and 20 times D-xylose reductase activity compared to that of the wild type strain (Table 2).

Table 2. *XyrA* copy number and D-xylose reductase activities in *A.niger* transformant strains.

Strain	Copy number	Activity (U/mg)
NW219	1	2
NW280	-	0.4
NW219::4400-3 (MC3)	3-4	9
NW219::4400-12 (MC12)	8-10	19
NW219::4400-19 (MC19)	15-20	38

To construct a *xyrA* disruption mutant, a 2.3 kb *Xho*I fragment containing the *pyrA* gene was subcloned in the opposite direction with respect to transcription, in a unique *Sal*I site of *xyrA* in pIM4400. The resulting plasmid, pIM4403 (Fig. 2B), was used in transformation of NW219 and transformants were selected for uridine prototrophy. Identification of the *xyrA* disruption mutant, NW280, was done by Southern blot analysis. The D-xylose reductase activity measured in the *xyrA* disruption mutant was decreased to 20 % of the level in the wild type

strain. The remaining activity results from the presence of L-arabinose reductase which is also able to convert D-xylose to xylitol (Witteveen *et al.*, 1989).

Role of D-xylose reductase in regulation of the expression of the xylanolytic system

D-xylose reductase activity potentially plays a role in the regulation of the expression of the xylanolytic enzyme system, as this activity might determine the rate at which the inducer D-xylose is metabolised. To investigate this, the *A.niger* wild-type, three *xyrA* multiple copy strains and a *xyrA* knock-out strain, a *xlnR* multiple-copy and *xlnR* knock-out strain (van Peij and de Graaff, unpublished communications) were grown in liquid culture. All these strains were precultured in MM containing 100 mM D-fructose as a carbon source and subsequently transferred to MM containing 50 mM D-xylose and to MM containing 1% beechwood xylan as inducing carbon sources. Transcription patterns of *xyrA,* the endoxylanase B encoding gene *xlnB* and the β-xylosidase encoding gene *xlnD* were analysed by Northern blot analysis (Fig. 4).

Transformants having multiple copies of *xyrA* showed an increase in *xyrA* transcript levels compared with the wild-type when grown on D-xylose and xylan. This increase is related to the number of *xyrA* copies integrated into the genome (Table 2). Increased *xyrA* transcription in multiple-copy strains resulted in a decrease in transcription of *xlnB* and *xlnD*. In the *xyrA* disruption strain, no transcript of *xyrA* was detected. Disruption of *xyrA* caused a slight increase in transcription of *xlnB* and *xlnD* after 6 h of growth on D-xylose. On xylan, this increase is stronger but occurs at a later stage.

In the *xlnR* disruption mutant, no transcript of any of the three genes was observed after growth on D-xylose and xylan. High-performance liquid chromatography (HPLC) measurements showed that, after 12 h of growth on D-xylose, there was still a low amount of D-xylose (2% of the initial concentration) left in the medium. D-xylose reductase activities in the *xlnR* disruption strain are of the same level as in the *xyrA*-disrupted strain (Table 3). Multiple copies of the *xlnR* gene resulted in slightly increased transcription levels of *xyrA* and *xlnD* and in a strong increase of the *xlnB* transcript level after growth on D-xylose. A similar but stronger effect was observed in case of growth on xylan. No increased D-xylose reductase activities were observed in the XlnR multiple-copy strain.

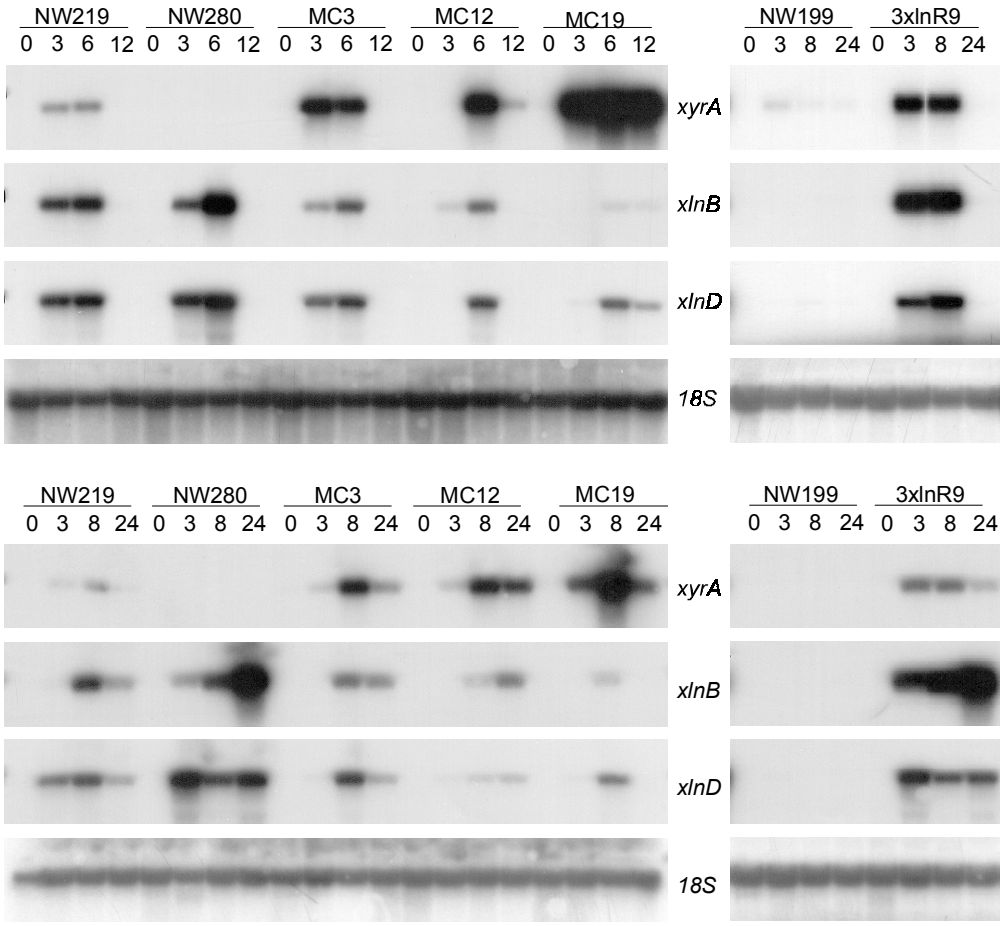

Fig.4. Northern blot analysis of *xyrA*, *xlnB* and *xlnD* transcription in different *A.niger* strains. All strains used were precultured in MM containing 100 mM D-fructose as a carbon source. For induction mycelium was transferred to 50 mM D-xylose and grown for 3, 6 and 12 h (A) and to MM containing 1% beechwood xylan and grown for 3, 8 and 24 h (B), after which the total RNA was isolated. The strains used are NW219 (wild type), NW280 (Δ*xyrA*) MC3, MC12, MC19, which contain respectively 3-4, 8-10 and 15-20 copies of the *xyrA* gene, NW199 (Δ*xlnR*) and 3xlnR9, which contains about 10 copies of the *xlnR* gene. Fragments used as probes for xlnB and xlnD are as described by van Peij et al. (1998b).

Table 3. D-Xylose reductase activities in wild type and transformant strains used in Northern blot analysis

Strain	Relevant feature	Time/activity (U/mg)			
		0 h	3 h	6 h	12 h
NW219	wild type	0.05	0.6	2.0	1.8
NW280	Δ*xyrA*	0.06	0.3	0.6	0.3
MC3	*xyrA* 3-4 copies	0.06	3.3	12.1	15.0
MC12	*xyrA* 8-10 copies	0.05	12.1	18.9	20.2
MC19	*xyrA* 15-20 copies	0.08	43.1	58.4	84.6
NW199	Δ*xlnR*	0.09	0.6	0.3	0.4
3xlnR9	multiple copy *xlnR*	0.08	0.4	1.7	2.0

To determine whether the decrease in *xlnB* and *xlnD* transcription levels results from titration of XlnR by the *xyrA* promoter or from an increased conversion of D-xylose due to increased D-xylose reductase activity, an expression construct was made in pUC18. In this construct the *xyrA* promoter is replaced by the strong constitutive promoter of the *A.niger* pyruvate kinase encoding *pkiA* gene (de Graaff *et al*., 1988). The resulting plasmid, pIM4411 (Fig. 2C), was used in co-transformation of the wild type strain NW219 using pGW635 as a selection marker. Multiple copy transformants, in which the transcription of *xyrA* is controlled by the *pkiA*-promoter, were selected by measuring the D-xylose reductase activity in cell lysates from 10 different transformants after 6h growth on D-xylose. Two transformants were selected having D-xylose reductase activity levels, which are comparable to MC12 and MC19 (Fig. 5B). Transcription levels of *xlnB* and *xlnD* were determined and were found to be similar to the wild type strain (Fig. 5A).

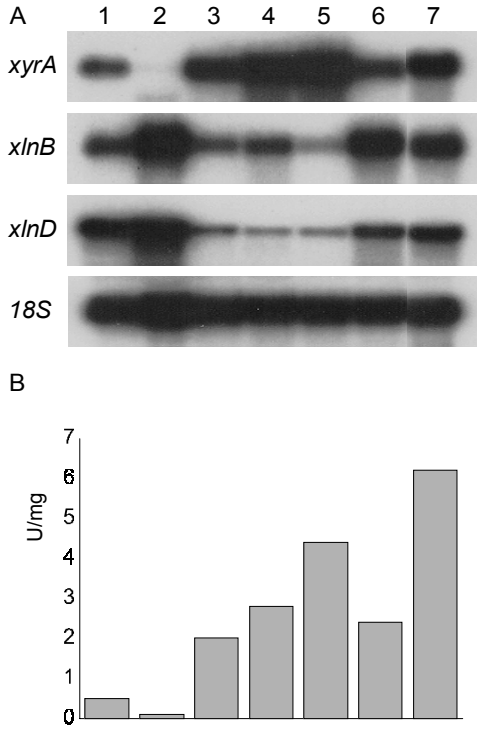

Fig. 5 Transcriptional analysis and D-xylose reductase activity in different *A.niger* strains. The strains were precultured in MM containing 100 mM D-fructose as a carbon source. For induction mycelium was transferred to MM containing 1% beechwood xylan. **A**. Northern blot analysis of transcription of *xyrA*, *xlnB* and *xlnD*. **B**. D-xylose reductase activities in cell free extracts. Lane 1 is: NW219 (wild type), lane 2: NW280 (Δ*xyrA*), lane 3: MC3, lane 4: MC12, lane 5: MC19 , which contain respectively, 3-4, 8-10 and 15-20 copies of the *xyrA* gene. Lane 6: TR5 and lane 7: TR12, both strains in which the *xyrA* gene is expressed under control of the *pkiA* promoter.

Discussion

Fungi have developed versatile mechanisms to adapt metabolism in response to environmental stimuli. In the absence of readily metabolisable carbon sources as *e.g.* D-glucose, but in the presence of polysaccharides such as xylan, *A.niger* is able to secrete a variety of enzymes to hydrolyse the xylan. As a result of xylan hydrolysis, a mixture of monomeric sugars, of which D-xylose is the main component, becomes available as a carbon source. Previous investigations have shown that the expression of xylanolytic enzymes by *A.niger* is controlled by the transcription activator XlnR (van Peij *et al*., 1998a,b). In addition, XlnR controls also the expression of other polysaccharidases as *e.g.* endo-glucanases (van Peij *et al*., 1998b), cellobiohydrolases (Gielkens *et al*., 1999) and galactosidases (de Vries *et al*., 1999).

Here, we have extended these studies by cloning cDNA fragments originating from XlnR controlled genes. This led to the isolation and identification of the D-xylose reductase encoding gene *xyrA*. In strains in which the *xyrA* gene is inactivated by gene disruption, the D-xylose reductase activity is not zero but strongly reduced, being about 20% of the wild type activity (Table 2). This is in agreement with the results found by Witteveen *et al*. (1989) who showed that L-arabinose reductase, although more active on L-arabinose, is induced on D-xylose as well. The residual D-xylose reductase activity in the strain in which the *xyr*A gene is inactivated, thus probably results from L-arabinose reductase.

From the results shown in Fig. 3, it is evident that the transcription of the *xyrA* gene is directly controlled by XlnR. Inactivation by gene disruption of *xlnR* not only leads to the loss of transcription of the extracellular xylanolytic genes, but also to loss of transcription of the *xyrA* gene. Also, the increased transcription levels found for *xyrA* as well as for *xlnB* and *xlnD* in the *xlnR* multiple copy strain, indicates coordinate regulation of genes encoding intracellular as well as extracellular enzymes involved in D-xylose metabolism. As a result, due to lack of xylan hydrolysing enzymes, XlnR loss-of-function mutants grow poorly on xylan as a carbon source (van Peij *et al.,* 1998b). However, these mutants are still able to grow on D-xylose as a carbon source, despite of the fact that there is no expression of D-xylose reductase. This can be explained by the presence of L-arabinose reductase, which has overlapping activity and thus compensates for the absence of D-xylose reductase.

In *A.niger* D-xylose is an inducer of the xylanolytic enzyme system. Because D-xylose reductase converts D-xylose to xylitol (Fig.1), increased D-xylose reductase activity may result in decreased expression of genes controlled by XlnR due to a decrease in inducer concentration. Northern blot analysis of *xlnB* and *xlnD,* encoding endoxylanase B and β-xylosidase, showed

that there is a decrease in transcript level of both genes in *xyrA* multiple-copy strains. However, *xyrA* multiple copy transformants under control of the *A.niger pkiA* promoter which have D-xylose reductase activity comparable to the XlnR controlled *xyrA* multiple-copy transformants, showed transcript levels of *xlnB* and *xlnD* that were the same as in the wild-type strain. Because XlnR does not control the *pkiA* promoter, it can be concluded that decreased expression of *xlnB* and *xlnD* in *A.niger* strains with multiple copies of *xyrA* is the result of titration of XlnR by the *xyrA* promoter.

Disruption of *xyrA* leads to an increase in transcription level of *xlnB* and *xlnD*. Due to the presence of L-arabinose reductase there is still conversion of D-xylose, but at a much lower rate because L-arabinose reductase accounts for only 20% of the D-xylose reductase activity. This might lead to higher intracellular D-xylose concentrations, which may cause two effects. Decreased D-xylose conversion might lead to higher intracellular inducer concentrations, causing an increased transcription. The other effect is in carbon catabolite repression, D-xylose also has, in addition to its inducing effect, a carbon catabolite-repressing effect (de Vries *et al*., 1999). The lower conversion of D-xylose may decrease carbon catabolite repression, leading to a higher level transcription.

In order to be able to use a polysaccharide as a carbon source, *A.niger* needs to secrete polysaccharide hydrolysing enzymes to its environment. In addition, the fungus also needs to adapt its carbon metabolism to the sugars released from the polymer. In *Klebsiella pneumoniae* and *Klebsiella oxytoca* it was found that the expression and secretion of pullulanase, which releases maltotriose from (α)1,6-glucosidically linked maltotriose units in pullulan, is under control of MalT, the maltose operon regulator (d'Enfert *et al.,* 1987, Pugsley, 1993). In *Saccharomyces cerevisiae*, *MEL1* encoding an extracellular α-galactosidase which converts melibiose to D-galactose and D-glucose, is under control of GAL4 (Lohr *et al*., 1995). Here, we show that in *A. niger* the transcriptional activator XlnR plays a central role, because it directs the transcription of genes encoding the hydrolytic enzymes involved in xylan degradation. The role of XlnR is not restricted to the control of the expression of the extracellular hydrolysing enzymes. By controlling the expression of the intracellular D-xylose reductase, it also plays a role in adjusting carbon metabolism to D-xylose catabolism.

Experimental procedures

Aspergillus niger strains, growth conditions and transformation procedure

All *Aspergillus niger* strains used were derived from N402, a low-conidiophore derivative from N400 (CBS 120.49) and they are listed in Table 1. All media used were based on minimal medium (MM) (Pontecorvo *et al*., 1953) and were, when appropriate, supplemented with 1 mM L-arginine, 1 mM histidine, 1.5 mM leucine, 2 mM lysine, 2 mM methionine, 8 μM nicotinamide and/or 5 mM uridine. *A.niger*, inoculated at 10^6 spores ml^{-1}, was grown in shake flasks (250 r.p.m.) at 30 °C using a starting pH of 6. In transfer experiments, mycelia were precultured for 18 h in MM using 100 mM D-fructose as carbon source, supplemented with 0.1% yeast extract and 0.1% casamino acids. The media used for induction contained 1% beechwood xylan (Sigma) or 50 mM D-xylose as inducing carbon sources. The mycelia were recovered and transferred as previously described (van Peij *et al*., 1998b).

In transformation experiments the recipient strain was *A. niger* NW219 (*cspA*1, *nicA*1, *leuA*1, *pyrA*6). Transformation was carried out as described before (Kusters-van Someren *et al*., 1991).

Construction and screening of the *A.niger* differential library

For the construction of the *A.niger* differential cDNA library, the cDNA of a XlnR loss-of-function mutant, NXA1-4 (van Peij *et al*., 1998a), was subtracted from the cDNA of the wild type strain N902 (Clontech PCR-Select cDNA subtraction kit). Both strains were pregrown for 18 h in MM using 100 mM D-fructose as a carbon source and then transferred to induction medium containing 50 mM D-xylose as an inducing carbon source. The mycelium was harvested after 6 h. The mRNA was isolated (Promega PolyATtract mRNA isolation systems) and cDNA was obtained using reverse transcriptase (Clontech Advantage cDNA PCR kit). After two rounds of subtractive hybridisation the cDNA was subjected to PCR to amplify the cDNA (Clontech PCR-Select differential screening kit). This cDNA was digested with *RsaI*, subcloned in pUC19 and transformed to library efficiency DH5α competent cells (Life Technologies). The resulting differential library was screened with forward- and reverse-subtracted cDNA probes according to the manufacturers instructions (Clontech). The forward-subtracted probe was made from the same cDNA used to construct the subtracted library. The reverse-subtracted probe was made by subtracting the cDNA of the wild-type strain against the cDNA of the loss-of-function mutant to reduce the background of housekeeping genes and high-abundant, differentially

expressed genes. In addition, the differential library was screened with a mixture of probes derived from genes those already known to be regulated by XlnR.

Cloning and sequencing

The vectors used in DNA cloning were pGEM (-T) (Promega), pBluescript (Stratagene), pUC18 and pUC19 (Yanisch-Perron *et al.*, 1985). *Escherichia coli* strains used in propagating plasmids and λ-phages were DH5a and LE392. Sequencing reactions were performed using the Thermo-Sequenase fluorescent-labelled primer cycle sequencing kit (Pharmacia-Amersham) using universal sequencing primers and the Cy5 Autoread sequencing kit (Pharmacia-Amersham) using gene-specific oligonucleotides. The sequencing reactions were analysed on an ALF*express* sequencer (Pharmacia). Database searches were performed using the National Centre for Biotechnology Information BLAST software. The *xyrA* nucleotide sequence has been submitted to the DDBJ/EMBL/GenBank databases under accession number AF219625.

Southern and Northern blot analysis

Fungal genomic DNA was isolated as previously described (de Graaff *et al*., 1988) and Southern blot analysis was performed according to standard methods (Sambrook *et al*., 1989) using the VacuGene XL Vacuum blotting system (Pharmacia). Total RNA was isolated from grinded mycelium using TRIzol reagent (Life Technologies). For Northern blot analysis, RNA was separated on a 1.6 % agarose gel in 10 mM sodium phosphate buffer pH 7 and, after capillary blotting to Hybond-N (Amersham), hybridised in 50 % formamide at 42°C. Filters were washed (1 x 5*SSC, 1 x 2*SSC and 2 x 0.2*SSC) under homologous hybridisation conditions at 68°C.

Enzyme assay

Preparation of cell-free extracts was done by re-suspending 0.5 g frozen mycelial powder in 1 ml extraction buffer containing 50 mM sodium phosphate buffer (pH 7), 5 mM $MgCl_2$, 0.5 mM EDTA and 5 mM β-mercapto-ethanol. After re-suspension the homogenate was directly centrifuged at 10,000 x *g* for 5 min. The D-xylose reductase assay was performed at 30°C as described by Witteveen *et al*. (1989) on a COBAS Bio auto-analyser (Roche). The protein concentration in the cell-free extract was determined using the bicinchoninic acid method (Sigma) following DOC-TCA precipitation.

Determination of the D-xylose concentration by HPLC

Culture filtrates were diluted in water to a final (estimated) concentration of 1 mM D-xylose or less. Analysis was done using a high-performance anion-exchange chromatography (HPAEC) system (Dionex Corp) equipped with a pulsed amperometric detector (PAD). Samples were loaded on a Carbopac PA 100 column (25 x 4 mm; Dionex) and eluted with 0.05M NaOH for 10 min at a flow of 1 ml/min. Concentrations were calculated from D-xylose standards of know concentration.

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CHAPTER 3

EglC, a new endoglucanase from *Aspergillus niger* with major activity towards xyloglucan

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Abstract

A novel gene, *eglC*, encoding an endoglucanase, was cloned from *Aspergillus niger*. Transcription of *eglC* is regulated by XlnR, a transcriptional activator that controls the degradation of polysaccharides in plant cell walls. EglC is an 858-amino-acid protein and contains a conserved C-terminal cellulose-binding domain. EglC can be classified in glycoside hydrolase family 74. No homology to any of the endoglucanases from *Trichoderma reesei* was found. In the plant cell wall xyloglucan is closely linked to cellulose fibrils. We hypothesize that the EglC cellulose-binding domain anchors the enzyme to the cellulose chains while it is cleaving the xyloglucan backbone. By this action it may contribute to the degradation of the plant cell wall structure together with other enzymes, including hemicellulases and cellulases. EglC is most active towards xyloglucan and therefore is functionally different from the other two endoglucanases from *A. niger*, EglA and EglB, which exhibit the greatest activity towards β-glucan. Although the mode of action of EglC is not known, this enzyme represents a new enzyme function involved in plant cell wall polysaccharide degradation by *A. niger*.

Introduction

Plant cell walls are composed predominantly of structural polysaccharides, which are associated in a matrix of cellulose microfibrils, hemicellulose polymers and pectin. Bacteria and filamentous fungi, including *Aspergillus* and *Trichoderma* species, can degrade plant cell wall polysaccharides efficiently by producing a mixture of extracellular hydrolytic enzymes.

The major component of plant cell walls is the β-1,4-glucan cellulose. Cellulose can be degraded by the coordinated action of cellulolytic enzymes, such as endoglucanase, cellobiohydrolase, and β-glucosidase. Most cellulolytic enzymes consist of a catalytic domain linked to a cellulose-binding domain (CBD) by a Pro/Ser/Thr-rich linker peptide. The cellulolytic enzyme system of *Trichoderma reesei* is the best-studied fungal example. This system contains five genes encoding endoglucanases, *egl*1 to *egl5* (14), two genes encoding cellobiohydrolases, *cbh1* and *cbh2*, (2, 19), and two β-glucosidase-encoding genes, *bgl1* and *bgl2* (1, 21). Endoglucanases cleave internal β-1,4-glucosidic bonds, while cellobiohydrolases cleave the disaccharide cellobiose from the either the nonreducing or the reducing end of the cellulose polymer chain (22). β-Glucosidases hydrolyze cello-oligosaccharides and cellobiose to D-glucose.

The expression of cellulases is controlled at the transcriptional level in both *T. reesei* and in *Aspergillus niger*. In the presence of D-glucose transcription is repressed, while in the absence of D-glucose and in the presence of cellulose certain oligosaccharides and/or disaccharide's (e.g. sophorose) transcription is strongly induced. In *T. reesei* D-glucose repression of transcription is mediated by Cre1, which also mediates repression of genes coding for enzymes involved in the degradation of hemicellulose (10, 12).

In *A. niger* two genes encoding endoglucanases, *eglA* and *eglB* (25), and two cellobiohydrolase-encoding genes, *cbhA* and *cbhB* (6) have been isolated and characterized. Both EglA and EglB lack a CBD and the associated linker region. CbhB has a catalytic domain and a CBD separated by a Ser/Pro/Thr-rich linker peptide, while CbhA has only the catalytic domain. Both enzymes release cellobiose upon incubation with carboxymethyl cellulose (CMC) (6).

We cloned the *xlnR* gene, encoding the transcriptional activator XlnR, in *A. niger* based on its ability to regulate the expression of genes encoding xylanolytic enzymes. However, XlnR controls both the transcription of genes encoding main and accessory xylanolytic enzymes and the transcription of the cellulolytic genes *eglA, eglB*, *cbhA,* and *cbhB* (25).

In this paper we describe a new endoglucanase, EglC, from *A. niger*. The enzyme is unique among the *A. niger* endoglucanases because it exhibits its greatest activity towards xyloglucan.

Results

Isolation and analysis of the *A. niger eglC* gene

We cloned a 3,959-bp *Cla*I genomic DNA fragment containing the *eglC* gene (accession no. AY040839) into pIM4490. This *Cla*I fragment was sequenced and contained 1,037 bp of the 5' noncoding region and 118 bp of the 3' nocoding region. In the promoter region, one putative XlnR binding site (5'-GGCTAA-3') (24) was found, 395 bp upstream of the ATG translation start codon. The coding region of *eglC* is 2,574 bp long and is interrupted by five introns. All five introns are located in the first 1,000 bp of the coding region, and they range from 43 to 49 bp long. The *eglC* open reading frame encodes a 858-amino-acid protein. EglC contains a putative N-terminal signal sequence (amino acids 1 to 19), a Ser/Thr-rich linker peptide (amino acids 744 to 814), and a C-terminal CBD (amino acids 814 to 846). Based on the deduced amino acid sequence, the molecular weight of EglC was estimated to be 90.5 kDa, and the isoelectric

point was estimated to be 4.1. A Blast search with the Swissprot database showed that EglC exhibited a high level of sequence homology (75% identity) with AviIII (accession no. AB015511) of *Aspergillus aculeatus* (20) (Fig. 1.).

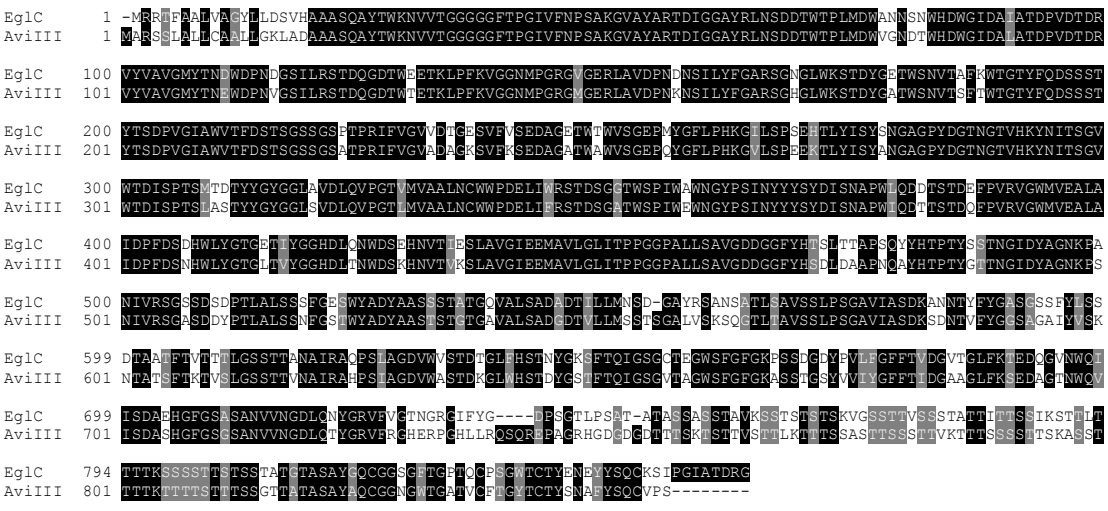

Fig. 1. Alignment of the amino acid sequences of EglC from A. niger (accession no. AY040839) and AviIII from *A. aculeatus* (accession no. AB015511) obtained by using ClustalW (http://www.ebi.ac.uk/clustalw) and Boxshade (http://www.ch.embnet.org). Identical amino acids at conserved positions are indicated by black boxes, similar residues are indicated by grey boxes.

Purification and characterization of EglC

We purified EglC from an overproducing *A. niger* strain. This strain was obtained by transforming *A. niger* NW188 (3) using a *xlnA*-promoter-*eglC* gene fusion (pIM4496) to increase *eglC* transcription. The purified protein was analyzed for activity with azo-CM-cellulose.

Maximum EglC activity towards azo-CMC occurred between pH 3.5 and pH 5, and the optimum pH was 4.5. The optimum temperature, at which the enzyme had the greatest activity, was 55 °C, as determined in 20 mM sodium acetate (pH 4.5). The stability of the enzyme was examined at the optimum pH (pH 4.5) in 20 mM sodium acetate buffer at 30 and 55°C. After incubation for 145 h at 30 °C, 90% of the activity remained. The enzyme was less stable at the optimum temperature (55°C) and optimal pH (pH 4.5); 50% of the activity remained after incubation for 48 h, and 20% of the activity after incubation for 145 h.

EglC is an endoglucanase

EglC has endoglucanase activity, since it exhibited activity towards CMC, azo-cellulose, β-glucan, and tamarind xyloglucan, but no activity was found towards xylan. EglC released oligosaccharides from xyloglucan (Fig. 2). Xyloglucan consists of a β-1,4-glucan backbone with substitutions of α-1,6-linked D-xyl *p* residues that are in turn variably replaced by β-1,2 linked Gal *p* residues (27). In tamarind xyloglucan, the Glc/Xyl/Gal/Ara ratio is 11:9:5:1 (Megazyme). However, the precise mode of action of EglC could not be determined based on the high-performance liquid chromatography (HPLC) patterns. EglC was most active towards xyloglucan (Table 1) (activity, 19 U/mg).

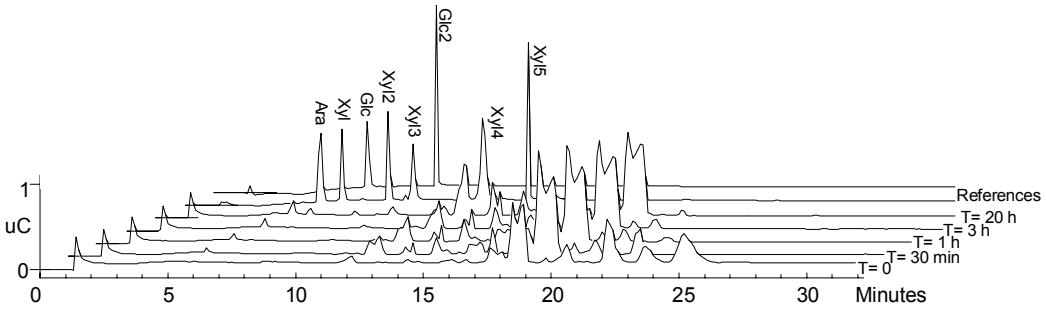

Fig. 2. HPLC analysis of EglC activity xyloglucan. The enzyme (12.6 μg) was incubated for 0.5, 1, and 3 h and overnight with 0.2% xyloglucan in 500 μl 20 mM sodium acetate (pH 4.5). Fifty microliters of an inactivated (5 min, 100 °C) reaction mixture that was diluted 20-fold in water was analyzed by high-performance anion-exchange chromatography using a Dionex system with a Carbopac PA-100 column and pulsed amperometrical detection with a 0.05 to 0.90 M NaOH gradient suitable for glucose oligosaccharide separation. The standards contain D-glucose (Glc), cellobiose (Glc2), L-arabinose (Ara), D-xylose (Xyl), xylobiose (Xyl2), xylotriose (Xyl3), and xylopentaose (Xyl5).

Table 1. Specific activities of EglA, EglB and EglC towards CMC, β-glucan and xyloglucan.

Enzyme	Sp act (U/mg of protein) towards the following substrates[a]		
	CM-cellulose	β-glucan	Xyloglucan
EglA	3 ± 0.4	59 ± 5	0
EglB	8 ± 0.6	22 ± 4	0
EglC	1 ± 0.2	1 ± 0.1	19 ± 1

[a]Specific activities were determined by using the reducing end group method (13) in triplicate.

Transcription of *eglC* is induced by XlnR and repressed by CreA

We compared the patterns of transcription of *eglC*, *eglA* and *cbhA* (Fig. 3). The *xlnB* gene was used as a reference. The levels of the *eglC* and *cbhA* transcripts were low in the wild-type strain. The *eglC* transcript was much higher in the strain containing multiple copies if *xlnR* than in the wild type. No *eglC* transcription was observed in the *xlnR* knockout mutant.

CreA represses xylanolytic gene expression in *A. niger* (3, 5). None of the genes in Fig. 3 were transcribed in the presence of D-fructose. In the presence of xylan *eglC* transcription increased in NW283, an *A. niger creA^{d}4* mutant strain relieved of carbon repression; this was also the case for transcription of *cbhA* and *eglA*.

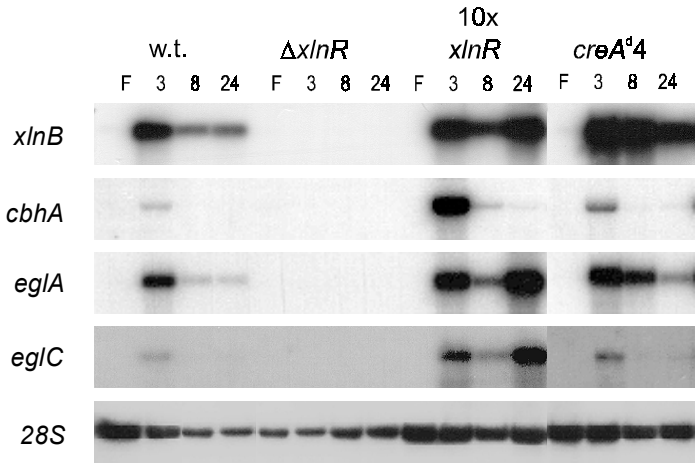

Fig. 3. Patterns of transcription of *xlnB*, *cbhA*, *eglA* and *eglC* in *A. niger* strains NW219 (w.t.), NW199 (Δ*xlnR*), N902::230-25.12 (10 x *xlnR*) (25), and NW283 (*creA^{d}4*). All of the strains used were precultured in MM containing 100 mM D-fructose (lanes F) as a carbon source. For induction, mycelium was transferred to MM containing 1% beechwood xylan and grown for 3, 8 and 24 h (lanes 3, 8, and 24, respectively), after which the total RNA was isolated. The fragments used as probes for *xlnB*, *cbhA* and *eglA* were fragments described by van Peij *et al* (25) and Gielkens *et al*. (6). For *eglC* an 850 bp *SalI-NcoI* fragment was used as the probe. The 28S rRNA serves as a loading control.

Discussion

We screened a differential library containing cDNA fragments derived from XlnR-regulated genes and isolated the *eglC* gene. *eglC* encodes an 858-amino-acid protein containing a C-terminal CBD attached to the catalytic domain via a Ser/Thr-rich linker peptide.

EglC is the third endoglucanase isolated from *A.niger* that is regulated by XlnR. The CBD of EglC (as well as *A. niger* CbhB and *T. reesei* CbhI) can be grouped into carbohydrate-binding module 1, previously known as CBD family I (carbohydrate-binding module

classification, http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html). This region contains approximately 40 amino acid residues and is found almost exclusively in fungi. The presence or absence of a CBD specifies the activity of the different cellulases from *T. reesei* (22). Removal or mutagenesis of the CBD from CbhI or CbhII reduces their activities on crystalline cellulose but not on soluble substrates (16, 23).

Based on its derived amino acid sequence EglC can be placed in glycosyl hydrolase family 74. A Blast search with the Swissprot database showed that no EglC homologue could be found in *T. reesei* or any other well-known cellulose-degrading fungi. However, a high level of sequence homology (75% identity) with *A. aculeatus* AviIII (accession no. AB015511) was found. It has been proposed that AviIII exhibits exoglucanase activity (20). Based on our data, EglC is an endoglucanase, and the enzyme is active towards azo-CMC, AZCL-cellulose, CMC, β-glucan and xyloglucan. The HPLC analysis showed that the enzyme degrades xyloglucan by an endo type of reaction.

The substrate specificity of EglA, EglB, and EglC towards β-glucan, CMC and xyloglucan have been determined. EglA and EglB exhibit the greatest activity towards β-glucan and minor activity towards CMC. No activity of EglA and EglB was found towards xyloglucan. The substrate specificity of EglC was different because the highest level of activity was towards xyloglucan and the lowest level of activity was towards β-glucan (Table 1). However, no synergy in enzyme activity was found when EglA, EglB and EglC were combined. *Trichoderma viride* produces an endoglucanase, endoIV, that has activity towards xyloglucan (26). Although *T.* viride endoIV and EglC both have xyloglucanase activity, we detected no significant sequence similarity between these proteins.

eglC transcription is regulated by XlnR, the transcriptional activator that controls genes encoding xylanolytic and cellulolytic enzymes in *A. niger*. Transcription of *eglC* in xylan-grown cultures is the same as for *xlnB*, *cbhA* and *eglA*. In the wild type, the level of *eglC* transcription is relatively low, but it increases in a strain containing multiple copies of *xlnR*. However, no transcripts are found in an *xlnR*-disrupted strain. Based on these results we concluded that XlnR regulates transcription of *eglC*. Although we found no putative CreA binding motifs in the *eglC* promoter, we did observe a slight increase in transcription of *eglC* in the *creA*^{d}*4* mutant. This result may indicate that CreA is involved in carbon catabolite repression of *eglC* since previous studies have shown that CreA-mediated carbon catabolite repression also plays a role under xylan-induced conditions (3, 4). Although transcription of all three endoglucanases of *A. niger* is regulated by XlnR, no synergy was found between the enzymes for degradation of β-glucan or xyloglucan was found (Table 2).

Table 2. Activity of EglA (62.5 ng), EglB (109 ng) and EglC (135 ng) towards β-glucan and xyloglucan.

Enzyme	Activity (nmol of reducing endgroups/min) towards[a]:	
	β-glucan	Xyloglucan
EglA	3.7 ± 0.3	0
EglB	2.4 ± 0.3	0
EglC	0.4 ± 0.1	2.6 ± 0.1
EglA + EglC	3.2 ± 0.4	2.7 ± 0.3
EglB + EglC	2.2 ± 0.1	2.9 ± 0.1
EglA + EglB + EglC	4.7 ± 0.2	2.8 ± 0.2

[a]Activities were determined by using the reducing end group method (13) in triplicate.

In plant cell walls, xyloglucan is closely linked to the cellulose fibrils. The *eglC* gene codes for an endoglucanase that has a high relative activity towards xyloglucan. EglC can cleave β-1,4-bonds between D-glucose units based on its activity towards CMC and β-glucan. This enzyme is probably more active on bonds that have D-xylose-containing side chains nearby in the chain. We assume that EglC binds to the cellulose chain by using its CBD, which enables it to efficiently degrade xyloglucan. Although no synergy was found towards EglA and EglB, EglC has its specific role in plant cell wall degradation. It is the first enzyme from *A. niger* described having activity towards xyloglucan.

Materials and Methods

A.niger strains and growth conditions

All of the *Aspergillus niger* strains used were derived from the wild type strain N400 (CBS 120.49). Strains used were NW188 (*goxC17*; *cspA1 pyrA6*; *leuA1*; *prtF28*,), NW219 (*cspA1 pyrA6*; *leuA1*; *nicA1),* NW199 (*fwnA6*; *goxC17*; *pyrA6* Δ*xlnR*::*argB*; *leuA5*), NW283 (*fwnA1*; *cspA1 pyrA6 lysA7*; *creA*^{d}*4*), and NW188::pGW635::pIM4496-22 containing an EglC expression construct.

All media had a pH of 6 and were based on *Aspergillus* minimal medium (MM) (15); the media were supplemented with carbon sources as indicated. Spores were inoculated at 10^6 spores ml^{-1}. In transfer experiments precultures grown with D-fructose were supplemented with 0.1% (wt/vol) Casamino Acids and 0.1% (wt/vol) yeast extract. After 18 h of growth,

mycelia were recovered by filtration over a Büchner funnel and washed with MM without a carbon source. These mycelia were transferred to MM containing various carbon sources.

Cloning and characterisation of *eglC*

While screening a differential cDNA library, constructed by subtracting the cDNA of a D-xylose-induced *A. niger* XlnR loss-of-function mutant from the cDNA of a D-xylose-induced wild type (9), we isolated a 300-bp *Rsa*I fragment that encoded the C-terminal portion of *eglC*. The complete *A. niger eglC* gene was recovered after screening an *A. niger* N400 genomic library in λEMBL4 (7). We used standard methods for other DNA manipulations, including Southern blotting, subcloning, DNA digestions, and λ phage and plasmid DNA isolation (18). Sequencing reactions were performed by using the Thermo-Sequenase fluorescently labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) using universal sequencing primers. The sequencing reaction mixtures were analyzed with an ALF*express* sequencer (Amersham Pharmacia Biotech).

Expression vector for *eglC* in *A. niger*

The *eglC* gene was fused to the promoter of the *Aspergillus tubingensis xlnA* gene (3) at its start codon using Splicing by Overlap Extension (SOE) as described by van Peij et al. (24), resulting in pIM4496. This plasmid included the coding region and a 3' noncoding flanking region of the *eglC* gene. Transformation of *A. niger* was performed as previously described (11).

Purification and characterisation of *A. niger* EglC

A. niger strain NW128::pIM4496-28 was precultured overnight using 2 liters of MM with 3% D-fructose and then was transferred to 2 liters of MM containing 50 mM D-xylose for 8 h. The mycelia were removed by filtration over a Büchner funnel, the culture was diluted with water so that the volume was 7.5 liters in order to decrease the salt concentration (<10 mM), and the pH was adjusted to pH 6 with 1 M NaOH. The culture filtrate was incubated in a batch preparation overnight at 4 °C with 20 ml Streamline DEAE (Amersham Pharmacia Biotech) that had been preequilibrated in 10 mM piperazine (pH 6) to bind protein. The supernatant was decanted, the Streamline DEAE was poured into a column, and EglC was eluted in eight 5-ml fractions with 1 M NaCl. The concentrated enzyme preparation was loaded on a 15.5-ml Source 30 Q column (Amersham Pharmacia Biotech) equilibrated with 10 mM piperazine (pH 6) and was eluted with a linear 0 to 1 M NaCl gradient. EglC eluted at an NaCl concentration of 0.3 M. The Source 30 Q enzyme pool was desalted and purified further by using a 313-ml Superdex 200 prep grade

column (gel permeation; Amersham Pharmacia Biotech) equilibrated with 10 mM piperazine and 100 mM NaCl.

The pH optimum of the purified EglC (3.7 ng) was determined by using 175 μl (final volume) portions of McIlvaine buffer at pHs varying from 2.9 to 7.5 (ionic strength equivalent to 0.5 M) with 200 μl AZO-CM-cellulose (Megazyme, Wicklow, Ireland) and incubation for 10 min at 40°C. The optimum temperature was determined by using 175-μl of 100 mM sodium acetate buffer at the optimum pH (pH 4.5) with 200 μl azo-CMC and incubation for 10 min at temperatures ranging from 25 to 80°C.

We tested EglC activity towards CMC (Sigma, St. Louis, Mo.), β-glucan (Megazyme) and tamarind xyloglucan (Megazyme) by incubating 2 μl of purified EglC (0.54 mg/ml) in preparations containing 200 μl of 0.5% substrate and 400 μl of 20 mM sodium acetate buffer (pH 4.5) for 10 min at 40 °C. Release of reducing end groups was measured at least two times in duplicate as previously described by Nelson-Somogyi (13). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmole of reducing end groups per min.

Purification of *A. niger* EglA and EglB

The endo-glucanases EglA and EglB were purified from culture filtrates of *Kluyveromyces lactis* CBS 2359 containing *eglA* and *eglB* expression constructs. The culture filtrates were diluted 1:10 in 10 mM piperazine (pH 6) and loaded on a 1-ml Mono Q column (Amersham Pharmacia Biotech) equilibrated with 10 mM piperazine (pH 6). Both enzymes were eluted using a linear 0 to 1 M NaCl gradient. EglA eluted at a concentration of 0.2 M NaCl and EglB at an NaCl concentration of 0.5 M. The presence of EglA or EglB in the fractions was determined by screening for β-glucanase activity on plates containing azurin-dyed and cross-linked (AZCL) β-glucan (Megazyme). The purified enzymes were desalted and analyzed by using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining.

We determined the activity of 1 μl of EglA (0.5 mg/ml) and 2 μl EglB (0.44 mg/ml) towards CMC, beechwood xylan (Sigma), β-glucan, and tamarind xyloglucan; we also determined the activities of these enzymes towards β-glucan and tamarind xyloglucan in combination with 2 μl EglC (0.504 mg/ml). Activity was determined by incubating the enzymes in 200 μl of 0.5% substrate in 400 μl of 20 mM sodium acetate buffer (pH 4.5) for 10 min at 40°C. The release of reducing end groups was measured as previously described by Nelson-Somogyi. (13).

Northern blot analysis

To verify that XlnR-controlled transcription of *eglC* occurred and to examine the effect of CreA repression on *eglC*, Northern blot analyses were performed. RNA was isolated from *A. niger* NW219 (wild type), NW199 (Δ*xlnR*), from *A. niger* N902::230-25.12, with 10 copies of the *xlnR* gene (25), and from NW283 a mutant (*creA*^{d}*4*) relieved of carbon repression (17). All strains were precultured in MM with 100 mM D-fructose as a carbon source and were then transferred to MM with 1% beech wood xylan and incubated for 3, 8 and 24 h.

Total RNA was isolated from powdered mycelia using TRIzol Reagent (Life Technologies, Rockville, Md.), according to the manufacturer's instructions. For Northern blot analysis, 10 μg of total RNA was glyoxylated and separated on a 1.6% (wt/vol) agarose gel (32). After capillary blotting to a Hybond-N membrane (Amersham Pharmacia Biotech), the transfer and the amount of RNA was verified by staining the RNA on the Hybond filter in 0.2% (wt/vol) methylene blue. Filters were hybridized at 42°C in a solution of 50% (vol/vol) formamide, 10% (wt/vol) dextran sulphate, 6x SSC (1x SSC is 0.15 M MaCl plus 0.015 M sodium citrate), 0.2% (wt/vol) ficoll, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) bovine serum albumin, 0.1% (wt/vol) SDS, and 100 μg of single-stranded herring sperm DNA (Life Technologies) per ml. The filters were washed in 0.2x SSC-0.1% (wt/vol) SDS at 65°C.

Nucleotide sequence accession number

The eglC sequence has been deposited in the GenBank and EMBL sequence databases under accession no. AY040839

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CHAPTER 4

Cloning and characterisation of the pathway-specific regulatory gene *xlnR*, which controls xylan degradation in *Aspergillus nidulans*

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Abstract

The *xlnR* regulatory gene of *Aspergillus nidulans* has been cloned and sequenced. Transformation of an *A. niger xlnR* knock-out strain using this gene restores xylanolytic enzyme production. An *A. nidulans xlnR* disruption strain produces virtually no xylanase or β-xylosidase activities under inducing conditions.

Results and Discussion

Hydrolysis of xylans is of considerable interest for various biotechnological applications (for reviews see 6, 42, 51). Natural xylan degradation by micro-organisms occurs through the co-ordinated action of various enzymes; endo-1,4-β-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) being the major components (46). While many filamentous fungi produce xylanolytic enzymes, the genetic amenability of *Aspergillus nidulans* makes this organism a convenient model for the basic study of the regulation of xylan-degrading enzymes (27).

A. nidulans, growing on xylan or xylose as carbon sources, secretes at least three xylanases of molecular masses 22, 24 and 34 kDa (12-15, 40) and one β-xylosidase (21), encoded by the *xlnA*, *xlnB*, *xlnC* and *xlnD* genes respectively (25, 38, 39). These genes are co-ordinately regulated by at least three independent regulatory mechanisms: (i) ambient pH regulation mediated by the wide-domain transcription factor PacC, (ii) carbon catabolite repression (CCR) mediated by the wide-domain repressor CreA and (iii) specific induction in the presence of xylan or xylose mediated by an as yet uncharacterised positive regulatory protein (26, 28, 34, 35, 39). *A. nidulans* xylanolytic genes are, consequently, an attractive model for the study of interactions between CCR, pH regulation and xylose induction. Recently different genes encoding Zn_2Cys_6 transcriptional activators involved in expression of xylanolytic and cellulolytic genes have been cloned from *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* (1, 31, 50). Studies of the regulation of the *A. oryzae xyn1F* promoter (encoding a major endo-xylanase) in *A. nidulans* (31) indicate that this organism has also a XlnR homologue that could mediate xylan induction.

To clone the *A. nidulans xlnR* gene we have screened, under heterologous conditions of hybridisation (43), an *A. nidulans* lambdaZAP genomic library with a DNA fragment of the *A. niger xlnR* gene (50). Positive plaques were purified and the corresponding phagemids containing the putative *A. nidulans xlnR* were excised. Restriction analysis of three phagemids showed the presence of a 5.7-kb *BamH*I fragment. This result correlated well with that found on

blots of *A. nidulans* genomic digests hybridised to the same probe (data not shown). Sequence analysis of the 5.7-kb *BamH*I insert reveals the presence of two open reading frames (ORF), one encoding the last 215 amino acids of a putative mitochondrial RNA polymerase (RPOM) (GenBank/EMBL/DDBJ accession number AJ544577) with a 33% identity to the RPOM of *N. crassa* encoded by the nuclear *cyt*-5 gene (5) and a second ORF encoding a protein highly homologous to the *A. niger* and *A. oryzae* XlnR regulatory proteins. Close linkage between these two genes is also found in *A. niger* (50).

The proposed ORF of the *A. nidulans xlnR* gene encodes a protein of 900 aa residues with a predicted molecular mass of 98.441 kDa. We postulated the AUG translation initiation codon based on *A. oryzae* XlnR data (31), although we have noticed the presence of a downstream in-frame AUG, also in a favourable context (2, 20), that would render a protein 25 amino acids shorter. In this regard, fusion experiments to the second AUG have shown the functionality of the shorter protein (Tamayo and Orejas, unpublished data). Two introns of 61 and 57 nucleotides, which follow the general characteristics of filamentous fungal introns (reviewed in 16), were identified by homology comparison to the translated product of the *A. niger xlnR* gene. The positions of the introns are conserved between *xlnR* genes from *A. niger* and *A. nidulans* (E119/F120 into the $Zn(II)_2Cys_6$ region and Q852/R853 in the *A. nidulans* encoded protein), though they differ in length and sequence.

Sequence analysis of the 1065 bp promoter region of *xlnR* ($xlnR_p$) revealed the existence of 11 consensus 5'-SYGGRG-3' target sites for the CreA repressor (8, 10), and two putative 5'-GCCARG-3' PacC binding sites (11, 48), suggesting that the transcription of the *xlnR* gene is regulated by these transcription factors. Moreover, the occurrence of such CreA target sites in $xlnR_p$ strongly suggests that direct repression of *xlnR* transcription might, at least in part, be responsible of the glucose repression of *xlnA* and *xlnB* genes indirectly mediated by CreA, as has been previously described (34, 35). General promoter elements such as TATA or CCAAT boxes are not found. In the 3' region, a putative polyadenylation signal (5'-AUAUAAA-3') is located 41 nucleotides downstream the proposed stop codon (16).

The deduced XlnR protein (Fig. 1) contains several features encompassing a Zn_2Cys_6 transcription activator (44), a family of proteins almost exclusively present in ascomycete fungi which prototype is the *Saccharomyces cerevisae* GAL4 protein. In its N-term (residues 98-124) the characteristic $Zn(II)_2Cys_6$ binuclear cluster DNA binding domain (DBD) (29, 30, 36) is found. Before the first cysteine, the ^{91}RRR^{93} motif, which is important in AlcR for DNA binding and function (33), is also present. Downstream this region, a potential nuclear localisation

sequence (NLS) ([128]RERKKRGKASKK[139]), similar to that of the antigen large-T from SV40 (18, 19), is found at residues 130-133.

```
A. niger          MSTPSIPQFT--SPFSPFS-SGSHSTGMAPSQTVGLDTMS-------------------- 37
A. fumigatus      MSTTSLQHFP--HSYSPFS-SSRSLNRMAQSQTSGLDTLAEGSQYALEQLQMSREAAGSG 57
A. oryzae         MSTTSIQHFT--SSFSPFS-SGTQPVGMAQSQTVGLDTLAEGSQYALEQLQLSREANGAS 57
A. nidulans       MSTTSLQQFATATSFSPFSNSQSARMSQSQSQTIGLDTLAEGSQ----------------- 44
N. crassa         MLSNPLHRFAPYHAMPSPTLLSGGHVTASHLHAAGLDTMGPGSH----------------- 44
                  * : .: :*.   . .. :         :  :: ****:.

A. niger          ---------------HTKDQPPFDNE----KNQSTGSGFRDALQRDPLVEARSAVRKTSS 78
A. fumigatus      EAT--------DSVGKPKDQFQVDNDN--HHNNHSLSNFKNPSQRDPLVEARSTIRKNSA 107
A. oryzae         AVDGGVPNPLRSSISKPQGQQLYSDESSAQHTQNATTGFRNLPQRDQLAEARSTIRKSSN 117
A. nidulans       ---------------YVLEQLQLSREG---GNSENNSTFKPSSVRDSLAEARSMIRKNSS 86
N. crassa         ---------------YALQQLQQHVSV---HNHHLARAGPQPKHRQHPYGP--VTRATGA 84
                                      *     .     .           *:    . :  * ..

A. niger          SAPVRRRISRACDQCNQLRTKCDGQHPCAHCIEFGLTCEYARERKKRGKASKKDLAAAAA 138
A. fumigatus      SAPVRRRISRACDQCNQLRTKCDGQNPCAHCIDFGLTCEYARERKKRGKASKKDLAAAAA 167
A. oryzae         SGPVRRRISRACDQCNQLRTKCDGQNPCAHCIEFGLTCEYARERKKRGKASKKDLAAAAA 177
A. nidulans       SAPVRRRISRACDQCNQLRTKCDGQNPCAHCIEFGLTCEYARERKKRGKASKKDIAAAAA 146
N. crassa         AGPIRRRISRACDQCNQLRTKCDGQHPCAHCIEFGLGCEYIRERKKRGKASRKDLAAQAA 144
                  :.*:*********************:******:*** *** **********:**:** **

A. niger          AATQGS------NGHSGQANASLMGERTSEDSRPGQDVNGTYDSAFESHHLSSQP---SH 189
A. fumigatus      AAAAAATNSGQPNGSSGKEDAALVGGHTSPDRRP--TINGRYDPAFEVPRNLN-----GS 220
A. oryzae         AVANNG-TAPTSNGNTSNDSVSSAKRHTPSDGQSTQEVSGRYDPNFDASRNLATAGQSQL 236
A. nidulans       AAGHQG----------------GMGNRSPTDRRLSQEPGGRYDSVLEASRVQS------- 183
N. crassa         AAAAAQ------LNGHKNPSQAGENDQSPPNRTESTTATKRASSLPIEHQTTS------- 191
                  *.           .   . . :    ::. :           ..     :    .

A. niger          MQHASTAGISGLHESQTAPSHSQSSLGTTIDAMHLNHFNTMNDSGRPAMSISDLRSL--- 246
A. fumigatus      AQHSEASGMVGMQNSQHLPPHSQSSMGGGLEGLPLNGYNGLNDSGRPSMPVPELQSLHML 280
A. oryzae         GQHSDMSGMAGMQGSQQTP-HSQPSLGGAIDAIHLNHFNTLNDSNRPQMSVPDLRSLQML 295
A. nidulans       --HLPANGLSSIHN--TQAAHSQPPLGSALDALHLNHFTQLNESGRSQMPVSDLRSLQIL 239
N. crassa         -NDKTMSDMS-EGSVRSQRTGSMDSIDLGAHQTHIASHPGAMDRDLESPAALDLSYGN-V 248
                   ..    .: .        . *  .:.   .   :  .    : .    .  :*

A. niger          ---------PPSVLPPQGLSSGYNASAFALVNPQEPGSP-ANQFRLGSSAENPTAPFLGL 296
A. fumigatus      HNSHTNPRSPSSILPHHRYNGGYNDSAYSLMNPQEPNSTSISHFRLGSSTENPPNSFLGL 340
A. oryzae         HPSGANTRSPSGALPPQGMNSGYNDGAYSLMNASEANHPSINQYRLGNSAENPPAPFLGL 355
A. nidulans       HNN----PRSPSALP-HGLN-AYNDNTFSLLNSQEPNTTSLNHFRLGNSTDNPSAQFLGL 293
N. crassa         HQEYHRQGMGAHLMNGASHHTPYGSNQAAMSNYPDLPYALHTQSPTGYSANTSSGFRIGA 308
                            .  :        *. .  :: *  :   .  .:   * *::...   :*

A. niger          SPP--------GQSPGWLPLPSPSPANFPSFSLHPFS-STLRYPVLQPVLPHIASIIPQS 347
A. fumigatus      SPP--------AQSPGWLPLPSPSPANFPSFSMASFS-TTLRYPVLHPVLPHIASIIPQS 391
A. oryzae         SPP--------AQSPGWLSLPSPSPANFASFSMPPFS-STLRYPVLQPVLPHIASIIPQS 406
A. nidulans       SPP--------AQSPGWLPLPSPSPANFPSFPMAPFSGTSLRYPVLQPVLPHIASIIPQS 345
N. crassa         SPLSAYPMAGGSTSPGWMNLASPPPQFAQHIPQPTYSHAQLRYPVLEPLLPHLGNLMPVS 368
                  **         . ****: *.**.*     :.  .:* : ******.*:***:..::* *

A. niger          LACDLLDVYFTSSSSSHLSPLSPYVVGYIFRKQSFLHPTKPRICSPGLLASMLWVAAQTS 407
A. fumigatus      LACDLLDVYFTSSSSSHLSPQSPYVVGYIFRKQSFLHPTKPRVCTPGLLASMLWVAAQTS 451
A. oryzae         LACDLLDVYFTSFSPSHLSPQSPYVVGYIFRKQSFLHPTKPRVCSPGLLASMLWVAAQTS 466
A. nidulans       LACDLLDLYFTSSSSSHLSPQSPYVVGYIFRKQSFLHPTKPRVCSPGLLASMLWVGAQTS 405
N. crassa         LACDLIDLYFASSSSAQMHPMSPYVLGFVFRKRSFLHPTKPRQCQPALLASMLWVAAQTS 428
                  *****:*:**:* *.::: * ****:*::***:********* * *.********.****

A. niger          EAAFLTSPPSARGRVCQKLLELTIGLLRPLVHGPATGEASPNYAANMVINGVALGGFG-- 465
A. fumigatus      DAPFLTSPPSARGRVCQKLLELTIGLLRPLIHGPAPGETSPNYAANMVINGVALGGFG-- 509
A. oryzae         DAAFLTSPPSARGRVCQKLLELTVGLLRPLIHGPAPGETSPNYAANMVINGVALGGFG-- 524
A. nidulans       DAPFLTSPPSARGRVCQKLLELTIGLLRPLIHGPALGEASPNYAANMVINGVALGGFG-- 463
N. crassa         DAPFLTSVPSARGKICQKLLELTVSLLKPLIHTPSE-EPSP--VSSPIVDGVALGGLGVA 485
                  :*.**** *****::********:.**:**:* *: .*.**. .:. :::******:*

A. niger          ----VSMDQLGAQS---SATGAVDDVATYVHLATVVSASEYKAASMRWWTAAWSLARELK 518
A. fumigatus      ----VSMDQLGAQS---SATGAVDDVATYVHLATVISASEYKAASMRWWTAAWSLARELK 562
A. oryzae         ----VSMDQLGAQS---SATGAVDDVATYVHLATVISASEYKAASMRWWTAAWSLARELK 577
A. nidulans       ----VSMDQLGAQS---TATGAVDDVATYVHLATVVSASEYKAASMRWWTAAWSLARELK 516
N. crassa         LPGSISMDALTGETGAFGAAGTLDDVVTYIHLATVVSASEYKGASLRWWNAAWSLARELK 545
                      :*** * .::    *:*::***.**:*****:******.**:***.**********
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```
A. niger          LGRELPPNVSHARQDGERDGDGEAD--KRHPPTLITSLGHGSGSSGINVTEEEREERRRL 576
A. fumigatus      LGRELPPNTPHARPDAERDGDPDADLSKRHPPPLITSMGHGPGNTIINITEEEREERRRL 622
A. oryzae         LGRELPPNAPQPRQDGEPEDDTDVDMSKRNLPPLITSVGGNSGSTILNVTEEEREERRRL 637
A. nidulans       LGRELPPNASQPGQDGERENEGDNP-SKRNQ-----SLHG--GNSNVNVTEEEREERRRL 568
N. crassa         LGREIPQNSPSMQNSGS-------------------ELDGEMGNIPGMITEEEREERRRI 586
                  ****:* * .    ... ... .    .   ..  :.:    *.    :**********:

A. niger          WWLLYATDRHLALCYNRPLTLLDKECGGLLQPMNDDLWQVGDFAAAA------------- 623
A. fumigatus      WWLLYATDRHLALCYNRPLTLLDKECEGLLQPMNDDLWQAGDFAT--------------- 667
A. oryzae         WWLLYATDRHLALCYNRPLTLLDKECEGLLQPMNDDLWQAGDFAGAT------------- 684
A. nidulans       WWLLYATDRHLALCYNRPLTLLDKECSQLLQPMNDDLWQAGDFPAAT------------- 615
N. crassa         WWLVYIVDRHLALCYNRPLFLLDIECDGLLQPMDDTDYQNGNFYAYTDPNVLASDPNTPA 646
                  ***:* .************ *** **  *****:*  :* *:*   :

A. niger          YRQVGPPVECTGHSMYGYFLPLMTILGGIVDLHHAENHPRFGLAFRNSPEWERQVLDVTR 683
A. fumigatus      YRQAGPPVECTGHSMFGYFLPLMTILGEIVDLQQARNHPRFGLAFRNSAECEAQVLEIAR 727
A. oryzae         YRQVGPQVECTGHSMFGFFLPLMTILGEIVDLQQAKEHPRFGRVFRNSADWDHQVLEITR 744
A. nidulans       YRAVGPPIECTGHSMFGYFLPLMTILGGIIDLQQAREHPRYGLTFRSGPDLDQYIMAITQ 675
N. crassa         ARHRGPSFVCTGHSIFGYFLPLMTILGEIVDLHHARNHPRFGVGFRSSREWDDQTAEITR 706
                   *  ** . *****::*:********* *:**::*.:***:*  **.. : :     :::

A. niger          QLDTYGRSLKEFEARYTSNLTLGATDNEPVVEGAHLDHTS-----------PSGRSSSTV 732
A. fumigatus      QLDVYAQSLKEFETRYTSSLALGAAETEAAMDGSHPNHVS-----------PSGRSSSTV 776
A. oryzae         QLDTYAQSLKEFEARYTSSLALGAGESEAAIEGSHLDHVS-----------PSGRSTSTA 793
A. nidulans       QLDAYGQSLKDFEARYINSLALAENEPP---ENPHIDHLS-----------PSGRSSSTV 721
N. crassa         HLEIYEESIKRFEHRNLSLSAQAQAADEKAAEAAGVPTANDVPHDAGTPSVQSVHSVHTT 766
                  :*: * .*:* ** *  .  : .       : .     .            * :*  *.

A. niger          GSRVSESIVHTRMVVAYGTHIMHVLHILLAGKWDPVNLLEDHDLWISSESFVSAMSHAVG 792
A. fumigatus      ESRVNESIVHTKMVVAYGTHIMHVLHILLAGKWDPINLLDDNDLWISSESFVAAMGHAVG 836
A. oryzae         GSRVNESIVHTKMVVAYGTHIMHVLHVLLAGKWDPINLLEDHDLWISSESFIAAMSHAVG 853
A. nidulans       GSRVNESIVHTKMVVAYGTHIMHVLYVLLAGKWDPINLLEDHDMWISSESFLAAMSHAVG 781
N. crassa         SSRMTESDIQTRIVMAYGTHVMHVLHILLTGKWDPINLLDDNDLWISSQGFITATGHAVS 826
                   **:.** ::*::*:*****:****::**:*****:***:*:*:****:.*::* .***.

A. niger          AAEAAAEILEYDPDLSFMPFFFGIYLLQGSFLLLLAADKLQGDASPSVVRACETIVRAHE 852
A. fumigatus      AAEAAAEILEYDPDLSFMPFFFGIYLLQGSFLLLLTADKLQGDASPSVVRACETIVRAHE 896
A. oryzae         AADAAADILEYDPDIT-MPFFFGIYLLQGSFLLLLAADKLQGDVSPSVVRACETIVRAHE 912
A. nidulans       AAEAAADILEYDPDLSFMPFFFGIYLLQGSFLLLLAADKLQGDANPSVVRACETIVRAHE 841
N. crassa         AAEAISNILEYDPGLEFMPFFFGIYLLQGSFLLLLIADKLQVEASPSVVKACETIIRAHE 886
                  **:* ::******.:  ****************** ***** :..****:*****:****

A. niger          ACVVTLNTEYQRTFRKVMRSALAQVRGRIPEDFGEQQQRRREVLALYRWSGDGSGLAL- 910
A. fumigatus      ACVVTLNTEYQRTFRKVMRSALAQVRGRLPEDFGEQQQRRREVLALYRWTGDGSGLAL- 954
A. oryzae         ACVVTLNTEYQRTFRKVMRSALAQVRGRMPEDFGEQQQRRREVLALYRWTGDGSGLAL- 970
A. nidulans       ACVVTLNTEYQRTFRKVMRSALAQVRGRVPDDFGEQQQRRREVLSLYRWTGDGTGLALS 900
N. crassa         ACVVTLNTEYQRNFSRVMRSALAQVRGRVPEDLGEQHQRRRELLALYRWTGDGTGLAL- 944
                  ************.* :************:*:*:***:*****:*:****:***:****
```

Fig. 1. Comparison of the deduced amino acid sequences from *Aspergillus* and *Neurospora* XlnR proteins. Numbers indicate amino acid positions. Dashes indicate a gap. Asterisks indicate an identical residue between all 5 proteins, whereas dots represent conservative changes. The $Zn(II)_2Cys_6$ binuclear cluster region is underlined and the cysteine residues are written in bold letters. The 'Arg-Arg-Arg-Leu-Trp-Trp' motif is marked by dot-line underlining and the putative NLS is underlined with dotted line. The three loss-of-function mutations described in the *A. niger* protein are indicated by bold underlined letters. Other features are described in the text. The comparison was done using the program CLUSTAL W (http://www.ebi.ac.uk/clustalw/). Data from *A. niger* and *A. oryzae* polypeptides are from van Peij *et al*., (1998) and Marui *et al*., (2002) respectively whereas *A. fumigatus* and *N. crassa* are obtained from The *Aspergillus fumigatus* Genome Database contig 1319:a (http://www.tigr.org/tdb/e2k1/afu1/index.shtml) and from The *Neurospora crassa* 3 Sequencing Project at the Whitehead Institute/MIT Center for Genome Research (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/), hypothetical protein NCU06971.1 within contig 3.405. To construct this Figure we move the putative AUG codon predicted for the *A. niger* protein thirty six amino acids upstream.

The transcription activation domain (TAD) generally correlates with acidic parts of these proteins, usually located at the C-term (24, 37). An acidic region, D755-D819, with a net charge

of –9 which may act as TAD is present in XlnR. Moreover a proline-rich region of 42 (30.9% P) amino acids is located in XlnR starting at P295. Proline-rich regions have also been implicated in transcriptional activation of human CTF protein (32). XlnR is also rich in S(T)PXX motifs, highly conserved in DNA binding proteins (47), containing 13 SPXX repetitions. A highly variable central region supposed to be involved in regulation and in effector recognition (41) is present in Zn_2Cys_6 proteins. Within this region, the VI putative inhibitory domain (residues 560-594) (44) containing the six residues ^{565}RRRLWW^{570} first identified in UaY (45) is clearly observed in *A. nidulans* XlnR. Many Zn_2Cys_6 activator proteins bind to DNA as homodimers through a coiled-coil dimerisation domain located at the C-terminus of the $Zn(II)_2Cys_6$ region. To locate putative dimerisation elements we have used the programs COILS (2.2) and PAIRCOIL (4, 23). While two putative coiled-coil regions that might be involved in dimerisation have been described in the *A. niger* and *A. oryzae* XlnR proteins (31, 50), clear coiled-coil structures are not detected in the *A. nidulans* XlnR protein. The highest probability found is 0.2 from R91 to R105 and from Q675 to E688, suggesting that *A. nidulans* XlnR could bind to its target sites as a monomer. In fact, asymmetric consensus target sites have been proposed for *A. niger* and *A. oryzae* XlnR binding (9, 31, 50).

The $Zn(II)_2Cys_6$ binuclear cluster motif of *A. nidulans* XlnR shows strong identity with the corresponding regions of *A. niger* and *A. oryzae* XlnR proteins (96 and 100% respectively) (31, 50) and the putative XlnR proteins from *Aspergillus fumigatus* and *Neurospora crassa* (96 and 92% identity respectively) (Fig. 1). Less identity is found with *T. reesei* ACEII (1) and other members of yeast and filamentous fungi Zn_2Cys_6 transcriptional activators (Fig. 2). In the loop regions between the six cysteines and in the flanking regions other residues conserved among Zn_2Cys_6 proteins (44) are present in XlnR: one alanine before the first cysteine, the very conserved proline in the third loop which functionality in DNA binding has been probed in GAL4 (17) and one lysine before the third cysteine. The aspartate residues succeeding the first and third cysteines seem also to be conserved.

Comparison of the deduced *A. nidulans* XlnR protein and those from *A. niger* and *A. oryzae* revealed strong conservation throughout the protein (73% identity) (Fig. 1). The three residues L685, L858 and Y898, needed for *A. niger* XlnR function (50), are conserved. Screening of the recently available genomic sequences of *N. crassa* and *A. fumigatus* has revealed the presence of putative *xlnR* orthologues (50% and 73% amino acid identity respectively to the *A. nidulans* protein).

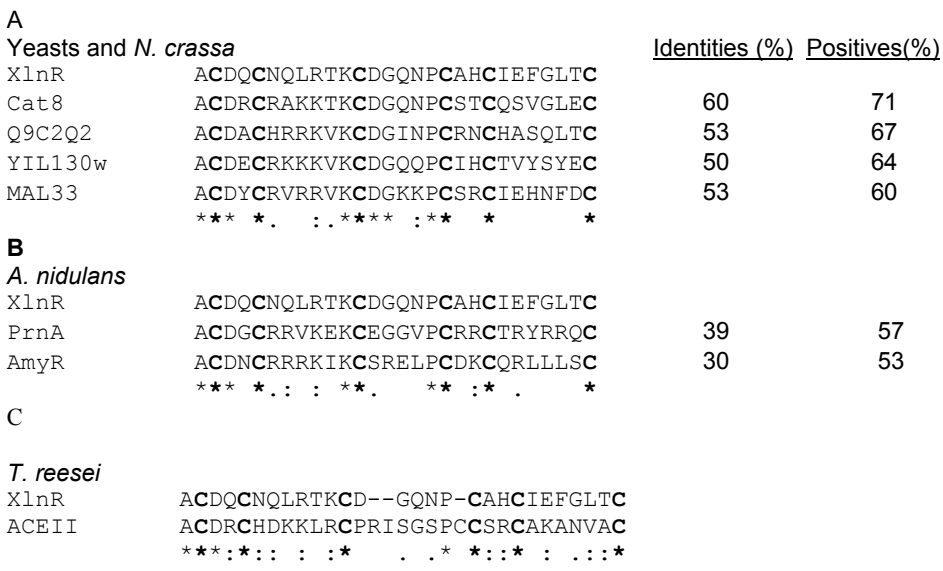

Fig. 2. Alignment of the $Zn(II)_2Cys_6$ region of XlnR with: (A) the corresponding domains of four fungal transcription activators most similar in amino acid sequence to XlnR, (B) two *A. nidulans* proteins from the same family of transcriptional factors that share identical spacing between cysteines and (C) ACEII from *T. reesei*, a novel activator of cellulase and xylanase encoding genes. Comparison was done with the CLUSTAL W program. Protein accession numbers in GenBank/EMBL databases are: *Candida albicans* Cat8 (AF222908), *N. crassa* Q9C2Q2 (AL513410), *S. cerevisae* ORF YIL130w (Z38059), *S. cerevisae* MAL3R (Z36166), PrnA (AJ223459), AmyR (AB024615), and ACE II (AF220671).

To test whether *A. nidulans* XlnR can functionally substitute *for A. niger* XlnR *in vivo*, we have co-transformed a *xlnR* loss-of-function mutation of *A. niger* with a plasmid containing the *A. nidulans xlnR* gene, including its flanking regions. Transformation was carried out as described previously (22). The expression of the gene is able to restore the $xlnR^+$ phenotype in an *A. niger xlnR* null strain (Fig. 3), indicating that the *A. nidulans xlnR* gene encodes a regulatory protein that *in vivo* binds and activates the *A. niger* xylanase and β-xylosidase encoding genes and suggesting that in *A. nidulans xlnR* is a pathway-specific activator of the xylanolytic system.

Reverse genetics was employed to further analyse the function of *xlnR* in *A. nidulans*. A *xlnR* null allele was made by replacing the 1.26-kb *Stu*I fragment, which contains the coding region for the first 292 amino acids as well as part of the 5' untranslated region, with the 3.2-kb *BamH*I/Klenow-filled *fragment* from pFB6 (3), which contains the *pyr*-4 gene from *N. crassa* (encoding orotidine 5'-monophosphate decarboxylase) that complements the *pyrG89* mutation of *A. nidulans* (strain A722). Transformation was carried out as described previously (49) and transformants were directly selected for growth on media lacking uridine. Single copy integrations at the *xlnR* locus were selected after Southern analysis of genomic DNA of pyr^+

strains for the predicted disruption restriction fragment pattern (data not shown). Two verified independent mutant strains, Δ*xlnR*-T5 and Δ*xlnR*-T37, were isolated.

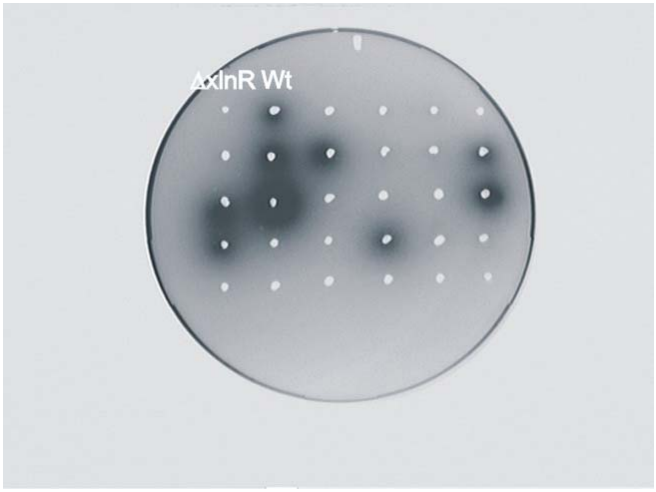

Fig. 3. Activity screening of transformants of *A.niger* Δ*xlnR* resulting from transformation using the *A.nidulans xlnR* gene. Transformants were selected and grown on minimal medium containing 1% Oat spelts xylan (Sigma) and 0.5 mM Methylumbelliferyl-β-D-xyloside (MUX)(Sigma). The MUX serves as a substrate for β-xylosidase and xylanases (low activity on this substrate). The plates were incubated for 15 hr at 30˚C and the activity was visualied by placing the plate on a transilliminator submitting light with a wavelength of 280

Whether disruption of *xlnR* affects the ability of the fungus to grow on xylan was studied by plating both *xlnR* disruptants and the host strain on minimal media containing glucose, xylose and xylan as the only carbon source. The disruptants are viable with no notable effect on growth rate on glucose and xylose (Fig. 4a). However, the *xlnR* deletion mutants showed a significant reduced growth on xylan medium compared to the growth of the host strain A722. While the wild-type strain produced clear halo, indicating substrate hydrolysis by secreted xylanase activities (Fig. 4a); very small halos were produced by the transformants. Thus, deletion of the *xlnR* gene confirmed that it is required for growth on xylan as carbon source and suggests that *xlnR* encodes a positively acting transcription factor which is necessary for the production of the xylanolytic enzymes. The residual growth could be due to a low level of XlnR-independent expression of the xylanolytic enzymes and/or the presence of accessory enzymes.

Transformants were also analysed in liquid cultures to determine if they were able to synthesise endoxylanases, β-xylosidase and α-L-arabinofuranosidase (ABF), an accessory xylanolytic enzyme. Enzyme production was tested by growth on minimal acidic media (7) containing 0.1% fructose and 0.5% casamino acids as the carbon and nitrogen sources respectively and transfers to the same media substituting the fructose by 1% xylan for 48 and 72

h. Both transformants lacked detectable xylanase activity compared to the wild-type strain. Similar results were also obtained when xylose was used as inducer. Zymograms of culture filtrates were made in order to detect different xylanase activities after SDS-PAGE (40). As can be seen in Fig. 4b, three bands corresponding to the three described xylanases X_{22}, X_{24} and X_{34} were detected under our experimental conditions in the wild-type sample, while no bands were detected in Δ*xlnR*-T5. Furthermore, β-xylosidase activity of the disruptants decreased greatly compared with the wild-type (Fig. 4c). However α-L-arabinofuranosidase activity did not decrease in the *xlnR* deletion strains either in xylan or under arabinose induced conditions (results not shown) indicating that this enzyme is not under the XlnR regulatory circuit, as was also previously shown for *A. niger* (50).

In conclusion, we present data showing that *A. nidulans* has a *xlnR* orthologue gene that encodes a Zn_2Cys_6 protein that controls the expression of the main enzymes involved in the degradation of the xylan backbone i.e. xylanases and β-xylosidase. Induction of some accessory enzymes such as ABF is not mediated by *xlnR*.

Nucleotide sequence accession number. The EMBL accession number for the *xlnR* sequence is AJ272537.

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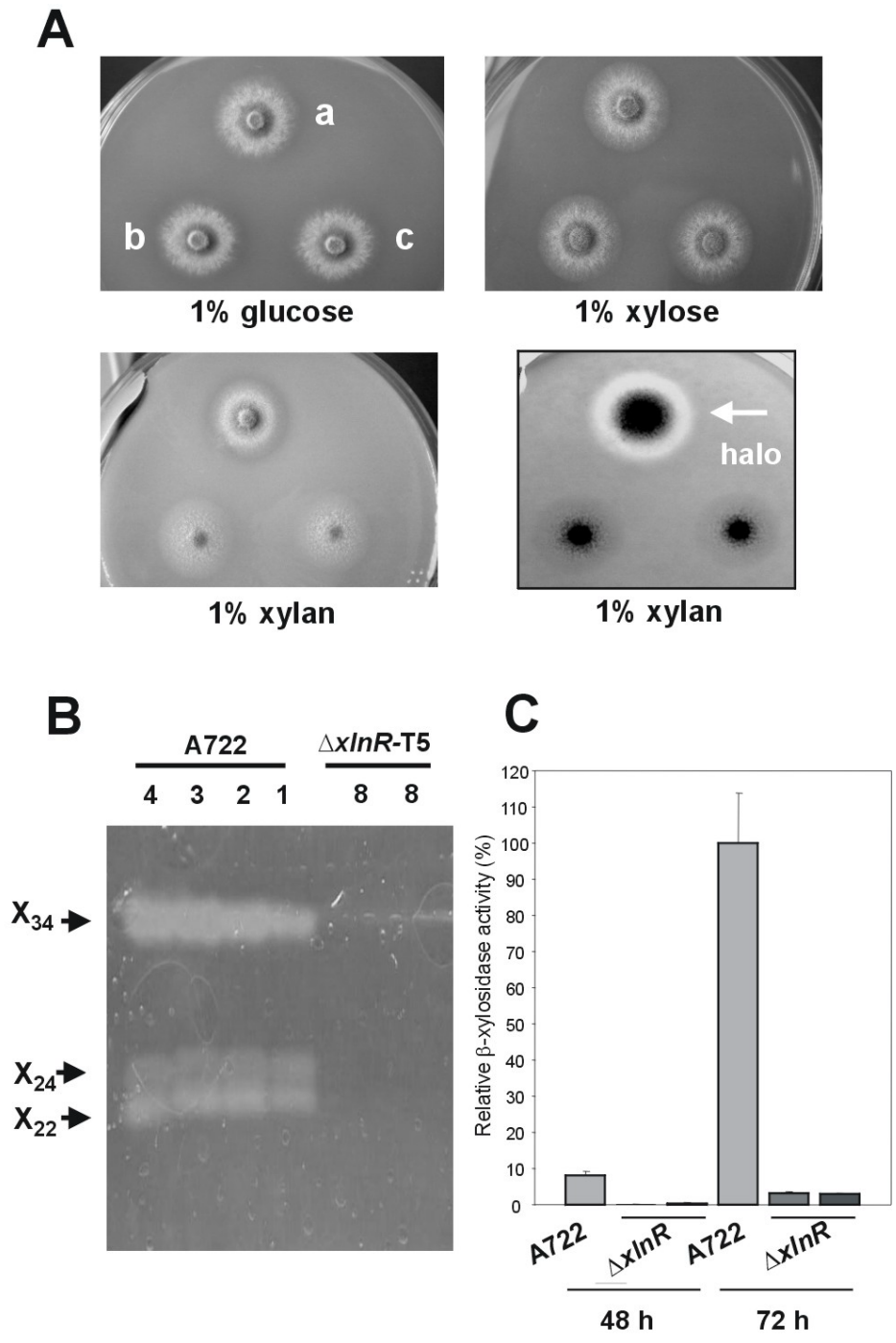

Fig. 4. Phenotypic characterisation of the *A. nidulans xlnR* disrupted strains. (A) Growth of the mutant strains Δ*xlnR*-T5 (b), Δ*xlnR*-T37 (c) and the host *xlnR*$^{+}$ GM191 strain (a) on plates containing 1% glucose, 1% xylose or 1% xylan as carbon source. (B) Zymogram analysis of extracellular xylanases produced by GM191 and Δ*xlnR*-T5. Numbers indicate micrograms of protein from culture filtrates loaded on the gel. (C) Bar diagram of the relative β-xylosidase activities in the GM191 strain and in both Δ*xlnR* mutants. Activities are presented as percentages of that observed in GM191 under induced conditions (1% xylan) at 72 hours. Values are presented as the average of three independent experiments and their standard deviation.

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CHAPTER 5

Regulation of the transcriptional activator XlnR from *Aspergillus niger*

Abstract

A mutant *Aspergillus niger* strain, NW147, which is constitutive for xylanase expression, was isolated. Sequence analysis of the x*lnR* gene of this strain, encoding the transcriptional activator XlnR, revealed that one single amino acid substitution, V756F, is responsible for the constitutive phenotype. Using N-terminal Gfp-fusions we showed that XlnR is present in the nucleus under inducing as well as non-inducing conditions. This implies that induction transcription of the structural genes is not regulated *via* nuclear transport of its transcriptional activator XlnR. It also indicates that the *xlnR* gene itself is not controlled at the transcriptional level. Introduction of the constitutive *xlnR* gene in a CreA derepressed strain suggested that there is a CreA-independent D-glucose inhibition mechanism acting at the XlnR protein. We hypothesise that mutation V756F in XlnR converts XlnR into an active state insensitive to the regulatory mechanism that converts it into an inactive state in the presence of a repressing carbon source.

Introduction

The *Aspergillus niger* transcriptional regulator XlnR activates the transcription of genes encoding cellulose and hemicellulose degrading enzymes (6, 10, 11, 24). XlnR belongs to the zinc binuclear cluster family of transcription factors exclusively found in fungi.

Previously, a promoter deletion analysis of the *Aspergillus tubingensis xlnA* gene in *A. niger* indicated that the XlnR-controlled enzyme system is regulated by a dual control mechanism. It was proposed that carbon catabolite repression by CreA takes place at two levels, directly by repression of transcription of the structural genes and indirectly by repression of transcription of the gene encoding the transcriptional activator (9). This would be similar to the double-lock mechanism described for the regulation of the *alcA* gene expression in *Aspergillus nidulans* (18).

Studies of Zn binuclear cluster transcription activators, however, have shown that in most cases this type of activator is regulated at the post-translational level. Examples are e.g. GAL4, LEU3 and PHO4 from *Saccharomyces cerevisiae*. In GAL4-mediated *GAL* gene expression there is direct interaction between two proteins GAL3 and GAL80. GAL3 interacts with GAL80 upon D-galactose induction to release GAL80 inhibition of GAL4 (2). LEU3, which is involved in branched-chain amino acid biosynthesis and ammonia assimilation, undergoes intramolecular changes mediated by α-IPM, which leads to unmasking of the

activation domain and subsequent activation of transcription (35). In case of PHO4, post-translational control occurs by means of nuclear localisation. In phosphate-rich media, PHO4 is phosphorylated and exported to the cytoplasm, thereby terminating expression of phosphate-responsive genes, whereas phosphate starvation leads to dephosphorylation and accumulation of PHO4 in the nucleus and subsequent transcription of phosphate-responsive genes (14).

To gain insight into the mechanism by which XlnR regulates transcription, we investigated the regulation of XlnR mediated activation of transcription, using a constitutive mutation in XlnR and a CreA derepressed mutant. This leads to a new hypothesis to explain the mechanism of D-glucose repression/inhibition of XlnR.

Results

A single amino acid mutation V756F in XlnR results in constitutive transcription of XlnR controlled genes

To isolate XlnR regulatory mutants we mutagenised an *A. nig*er strain containing a construct in which the *pyrA* gene is controlled by a fragment of the *A. tubingensis xlnA* promoter. This promoter fragment only contained an upstream activating sequence (UAS), upstream repressing sequences (URS) were removed (9). Mutants were selected for uridine prototrophy on plates with D-glucose and D-glucose/xylan as a carbon source. This resulted in the isolation of NW147, a mutant with a constitutive phenotype for xylanase expression.

The *xlnR* gene from NW147 was cloned by PCR and its nucleotide sequence was determined. Three mutations were found, a T->C transition in the promoter region 142 bp upstream the start codon, and two mutations in the coding region of XlnR: D651G and V756F. Since the *xlnR* clone from NW147 was obtained *via* PCR, errors due to amplification are a possibility. Therefore mutations found in the coding region, D651G and V756F, were separately introduced in XlnR *via* site-directed mutagenesis and introduced by transformation into an *A. niger xlnR* disrupted strain. Transformants obtained were tested for a constitutive phenotype. The transformants of the plasmid carrying the XlnR V756F mutation showed restoration of constitutive β-xylosidase activity and those containing the XlnR: D651G mutation did not.

To confirm that mutation V756F in XlnR results in an constitutive phenotype, a Δ*xlnR* strain harbouring a single copy of the mutated *xlnR* gene (*xlnR::V756F*) was selected and subjected to northern blot analysis. The transcription of the XlnR-controlled genes *xlnB*, *xlnD*, and *xyrA*, in this strain was found to be similar to that from NW147 (Fig. 1). In both strains,

transcription of the genes was found in cultures grown overnight on the non-inducing carbon source D-fructose. Thus, mutation V756F in XlnR leads to constitutive transcription of its target genes, meaning that there is transcription in the absence of inducer.

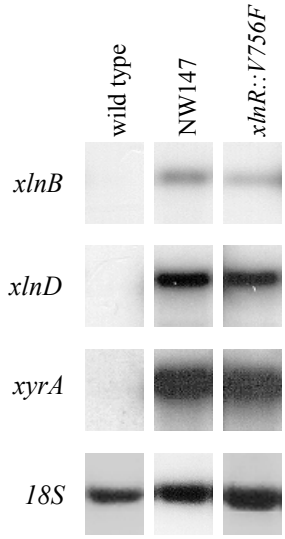

Fig. 1. Northern blot analysis of *xlnB*, *xlnD*, and *xyrA* transcription in *A.niger* wild type strain NW219, strain NW147 constitutive for xylanase activity and NW199::pIM4449, the Δ*xlnR* strain containing *xlnR:V756F*. The strains were grown overnight in MM containing 100 mM D-fructose as a carbon source.

Mutation V756F in XlnR affects transcription regulation *via* XlnR at post-transcriptional level

An amino acid mutation in XlnR leading to constitutive transcription of its structural genes suggests that XlnR activity is controlled post-transcriptionally. To investigate whether the constitutive xylanase transcription in mutant *xlnR::V756F* results from a post-transcriptional mechanism, mutation V756F was introduced in a construct in which the *xlnR* gene is controlled by the *pkiA* promoter (*pkiA(p):xlnR*). The *pkiA* gene encodes the glycolytic enzyme pyruvate kinase and is constitutively expressed (8). This construct was used to transform strain NW199 (Δ*xlnR*) and transcription levels of the *xlnB* gene in this strain (*pkiA(p):xlnR:V756F*) and two control strains harbouring a wild type *xlnR* gene were compared. The strains were grown on a combination of D-glucose and xylan to examine transcription under repressing conditions. Figure 2 shows that there is no transcript of *xlnB* independent whether the wild type *xlnR* gene is controlled by its native promoter or by the constitutive *pkiA* promoter (lane 1 and 2). When mutation V756F is introduced in *xlnR* controlled by the *pkiA* promoter (lane 3), there is strong transcription of *xlnB*. Thus, since in mutant (*pkiA(p):xlnR:V756F*) there is no control of *xlnR* at transcriptional level, we conclude that the constitutive effect of mutation V756F is due to a post-transcriptional mechanism.

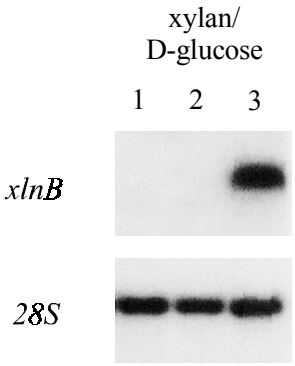

Fig. 2. Northern blot analysis of *xlnB* transcription in *A. niger* strains: 1. *xlnR(p):gfp:xlnR* (NW199::pIM4466); 2. *pkiA(p):gfp:xlnR* (NW199::pIM4474); and 3. *xlnR(p):gfp:xlnR:V756F* (NW199::pIM4476). All strains were precultured in MM containing 100 mM D-fructose and transferred to 50 mM D-glucose and 1% xylan for 4 h. Fragments used as probes are as described in Table 2.

XlnR is localised in the nucleus under inducing and repressing conditions.

Different post-transcriptional control mechanisms play a role in regulation of the gene expression in fungi. Post-transcriptional control can occur at the level of mRNA processing (1) or by controlling the stability of the mRNA (25). Also, in some cases control of gene expression takes place post-translationally, for example by controlling the protein stability, by regulated proteolytic processing, by modifying the protein activity through intra- or intermolecular interactions, or *via* protein localisation (29). The latter mechanism of regulation was found for PHO4 from *S. cerevisiae* and Cre1 from *Sclerotinia sclerotiorum* (14, 31).

To investigate whether subcellular localisation of XlnR plays a role in XlnR controlled regulation we used a reporter fusion protein of XlnR with Gfp (5). The *gfp* gene was fused 5' to *xlnR* transcriptional controlled by its own promoter (*xlnR(p):gfp:xlnR*). As a control, a *gfp:xlnR* construct controlled by the *pkiA* promoter (*pkiA(p):gfp:xlnR*) was used. These constructs were introduced in *A. niger* strain NW199 (Δ*xlnR*). The transformants were screened for recovery of xylanase activity and, based on the intensity of the fluorescence, selected for analysis. The Gfp-XlnR fusion proteins were localised using confocal microscopy. As shown in Figure 3, XlnR protein expressed from either its own or the *pkiA* promoter is retained in the nucleus under inducing conditions (in the presence of D-xylose) as well as under repressing conditions (in the presence of D-glucose). Counter-staining with DAPI showed strong staining of the areas in which the GFP-XlnR fusion protein was present. We conclude that XlnR is constitutively present in the nucleus and not transported to the nucleus in response to inducer.

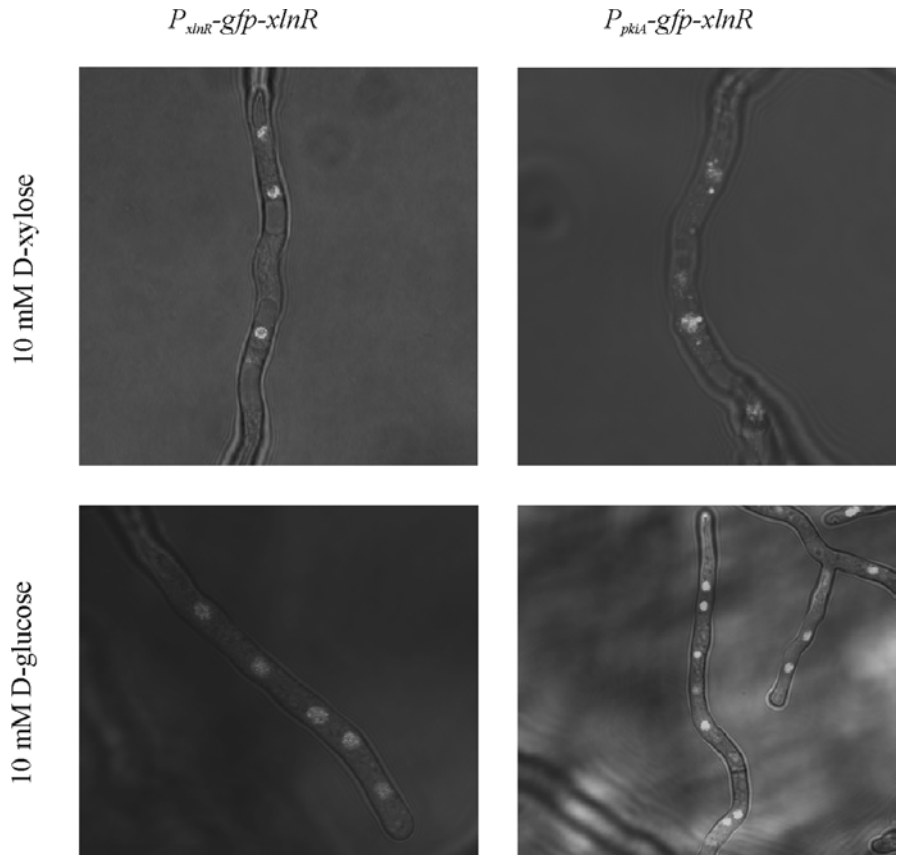

Fig. 3. Confocal microscopy of the GFP-XlnR fusion protein in *A. niger* strains *xlnR(p):gfp:xlnR* (NW199::pIM4466) and *pkiA(p):gfp:xlnR* (NW199::pIM4474) grown in cover glasses containing MM with 10 mM D-xylose (A) or 10 mM D-glucose (B) for 24 h. Magnification is 4-5 × 40.

D-glucose represses the transcription of XlnR-controlled genes *via* two different mechanisms

Carbon catabolite repression (CCR) *via* CreA plays a regulatory role by repressing transcription of the structural genes that are controlled by XlnR (6, 9, 34). In previous experiments it was shown that transcription repression of the xylanolytic genes occurs at two levels (9). To explain this finding it was suggested that in XlnR-controlled regulation of transcription, CreA interacts both at the level of the structural genes and at the level of the transcriptional activator (Fig. 4), similar to the AlcA/AlcR model from *A. nidulans* (17, 15). Now we have found that the *xlnR* gene is not regulated at transcriptional level. This implies that the mechanism of CCR that acts *via* XlnR are different from the CreA mediated CCR mechanism that controls the structural genes and acts at the level of transcription.

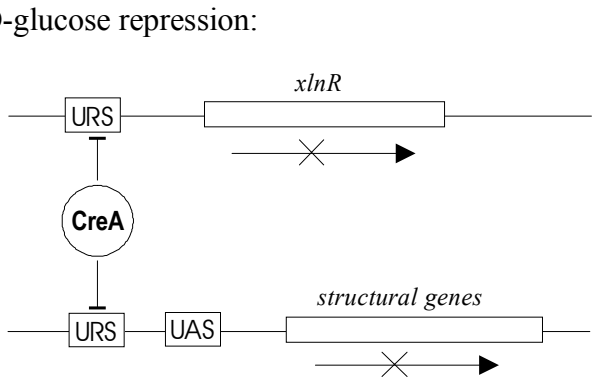

Fig. 4. Model for the "double-lock" mechanism for transcription regulation of the xylanolytic enzyme system in *A. niger* as proposed by de Graaff et al. (1994) (9). In this model the carbon catabolite repressor CreA regulates the transcription of *xlnR* gene encoding the transcriptional activator XlnR as well as its structural genes.

To examine D-glucose repression in XlnR-regulated transcription, the constitutive mutation V756F in XlnR (XlnR^{c}) was introduced as single copy integration in the *pyrA* locus, in a CreA-derepressed mutant (CreA^{d}) (28) that has a disrupted *xlnR* gene (XlnR^{-}). RNA was isolated from this strain, XlnR^{c}/CreA^{d}, and a number of control strains (Table 1). The transcription patterns of a selection of XlnR regulated genes (Table 2) were analysed by northern blot analysis (Figs. 5 and 6).

In the absence of inducer, with D-fructose as a carbon source, in the wild type strain (XlnR^{+}/CreA^{+}) no transcript was found for any of the XlnR-controlled genes, while after transfer to xylan all genes probed were transcribed (Fig. 5, panel A). Like in our previous studies, no transcripts of the XlnR controlled genes were found in the XlnR^{-}/CreA^{+} strain (Fig. 5, panel B) (24, 10). In the constitutive mutant NW147 and its control strain XlnR^{c}/CreA^{+}, all XlnR-controlled genes were transcribed at low level in the absence of inducer (Fig. 5, panels C and D). After induction by xylan all genes investigated were transcribed in the wild type and the two constitutive mutants. The difference in induction patterns of the wild type and the constitutive mutants (Fig. 5, panels C, D) can be explained in the following way. Increased transcription in the wild type strain (Fig. 5, panel A), as compared to the constitutive mutants in an early stage of induction, results from carbon derepression of transcription since initially only low amounts of D-xylose are released. After 8 h of induction, xylanolytic enzyme activity leads to higher D-xylose levels, which in turn leads to carbon catabolite repression of the structural genes *via* CreA (34). The increase in transcription in the constitutive mutants after 24 h of induction might result from exhaustion of the carbon source, leading to carbon derepression. The transcription patterns in NW147 and XlnR^{c}/CreA^{+} (Fig. 5, panels C, D) were essentially the same, minor differences are related to differences in the amount of RNA loaded.

In the CreA derepressed strain ($XlnR^{+}/CreA^{d}$) (Fig. 5, panel E), no transcript of the XlnR target genes was found on D-fructose. However, after transfer to inducing medium with xylan, strongly increased transcription was found compared to the wild type. This confirms that there is inducer-mediated CreA repression of XlnR regulated genes. In the $XlnR^{c}/CreA^{d}$ double mutant (Fig. 5, panel F), high transcript levels were found on D-fructose. In the $XlnR^{+}/CreA^{d}$ strain (Fig. 5, panel E), the lack of transcription on D-fructose has two reasons; there is no active XlnR since there is no inducer present in the medium, and there is a CreA-independent repression of the structural xylanolytic genes.

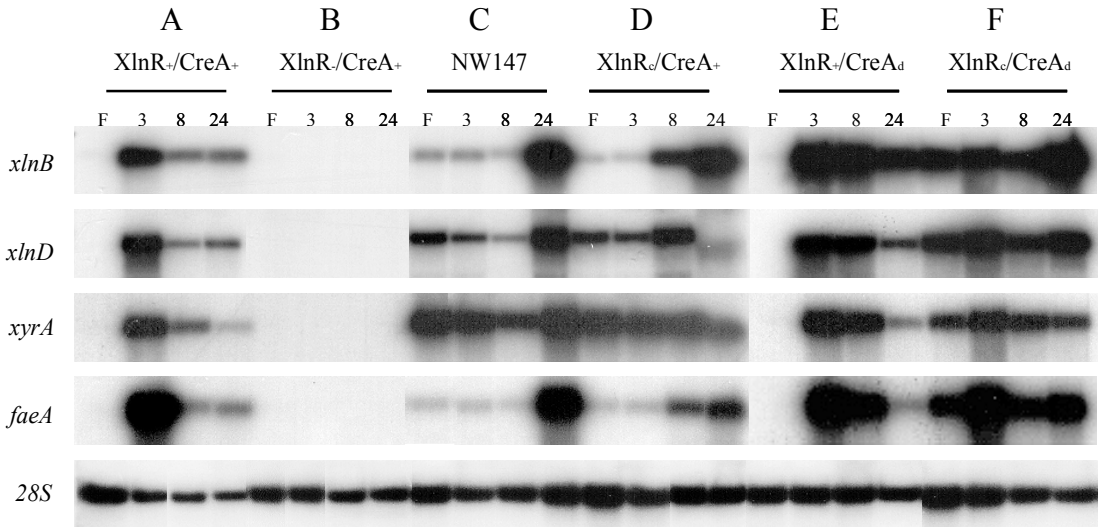

Fig. 5. Northern blot analysis of *xlnB*, *xlnD*, *xyrA*, and *faeA* transcription in different *A.niger* strains. All strains used were precultured in MM containing 100 mM D-fructose as a carbon source. For induction mycelium was transferred to MM containing 1% beechwood xylan and grown for 3, 8 and 24 h, after which the total RNA was isolated. The strains used are wild type strain NW219 ($XlnR^{+}/CreA^{+}$), Δ*xlnR* strain NW199 ($XlnR^{-}/CreA^{+}$), NW147 ($XlnR^{c}/CreA^{+}$), mutant strain harboring *xlnR:V756F* (NW199::pIM4449-24) ($XlnR^{c}/CreA^{+}$), CreA derepressed strain NW283 ($XlnR^{+}/CreA^{d}$), and CreA derepressed strain harboring *xlnR:V756F* (NW283::pIM4449-7) ($XlnR^{c}/CreA^{d}$). Fragments used as probes are as described in Table 2.

To distinguish between repressing conditions in the presence or absence of inducer, a selection of strains was grown on D-glucose as well as on a combination of D-glucose and xylan (Fig. 6). In the wild type strain (no.1) no transcription was found when grown on D-glucose or D-glucose+xylan. In the constitutive strain NW147 (no2) and its control (no.3), the level of transcription on D-glucose and D-glucose+xylan is essentially the same. In the CreA derepressed strain (no.4) grown on D-glucose also no transcription is found as in the wild type, but on D-glucose/xylan there is a low level of transcription. Apparently, in the CreA derepressed

strain, an inducing carbon source does not result in full transcription in the presence of a repressing carbon source. This suggests that there is a D-glucose repression mechanism in addition to CreA. In the double mutant XlnR^{c}/CreA^{d} (no.5), equally high transcript levels are found after transfer to D-glucose and D-glucose/xylan medium. The explanation for our result is that mutation V756F in XlnR abolishes the CreA-independent D-glucose inhibition mechanism that results in repression of transcription of the structural genes in a CreA derepressed strain and at the same time results in an inducer-independent active form of XlnR.

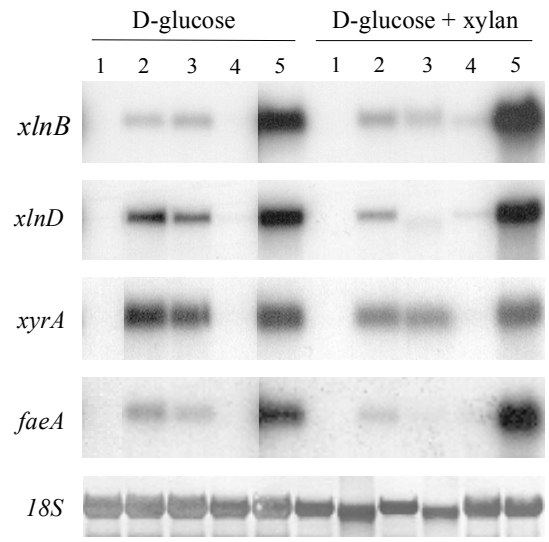

Fig. 6. Northern blot analysis of *xlnB*, *xlnD*, *xyrA*, and *faeA* transcription in different *A.niger* strains. All strains used were precultured in MM containing 100 mM D-fructose as a carbon source. For induction mycelium was transferred to MM containing 50 mM D-glucose, and 50 mM D-glucose plus 1% beechwood xylan and grown for 8 h, after which the total RNA was isolated. The strains used are; no.1: wild type strain (NW219); no.2: constitutive strain (NW147) no.3: mutant strain harboring *xlnR:V756F* (NW199::pIM4449-24); no.4: CreA derepressed strain (NW283); and no.5: CreA derepressed strain harboring *xlnR:V756F* (NW283::pIM4449-7) Fragments used as probes are as described in Table 2.

Discussion

To investigate the mechanism by which XlnR regulates transcription in *A. niger*, a mutant strain NW147, that is constitutive in xylanase activity, was isolated. Molecular analysis of the mutation in the *xlnR* regulatory gene revealed that mutation V756F in XlnR was responsible for the constitutive phenotype of the mutant. Apparently, one single amino acid substitution in XlnR results in transcription of its structural genes in the absence of inducer. This indicates that there is a regulatory mechanism that operates at the level of the XlnR protein *via* a post-translational mechanism.

Transcriptional activators from the Zn(II)2Cys6 binuclear cluster family are in many cases regulated *via* post-translational control mechanisms. In *S. cerevisiae* by example, GAL4 is

regulated by repressor binding (2), LEU3 is regulated *via* unmasking of its activation domain (35) and PUT3 shifts from an inactive to an active state *via* conformational changes (3). Regulation of transcription can also take place by means of nuclear localisation of the transcription factor, as is the case for PHO4 (14). Using Gfp-XlnR protein fusions, we showed that XlnR is present in the nucleus under inducing as well as non-inducing conditions. This is similar to the *A. nidulans* transcriptional activator PrnA that mediates induction of Proline utilisation (27). Also the lysine-biosynthetic transcription factor LYS14 from *S. cerevisiae* is localised in the nucleus under inducing and non-inducing conditions (4). Whether XlnR is bound to its target promoters as is described for PUT3 (3), or if inducer is necessary for binding, as is described for PrnA (7) and NirA (20) remains to be investigated. The finding that XlnR is localised in the nucleus under both inducing and non-inducing conditions indicates that the *xlnR* gene is not regulated at the transcriptional level.

In a promoter deletion analysis of the *A. tubingensis xlnA* gene done by de Graaff *et al*. (1994) (9) it was found that xylanase *xlnA* is also repressed *via* a sequence that contains no putative CreA sites. Therefore it was suggested that the xylanolytic system in *A. niger* is controlled directly by CreA at the level of the structural genes and indirectly at the level of the transcriptional activator. This model is analogous to the "double-lock" mechanism described for the ethanol regulon in *A. nidulans* (15, 18). However, the results of our study show that XlnR is not regulated at transcriptional level. Thus, the indirect repression of the XlnR structural genes in the "double-lock" mechanism proposed by de Graaff *et al*. (1994) does not occur at the level of *xlnR* transcription repression *via* CreA. Since under repressing conditions, mutation V756F in XlnR relieves the indirect repression of the structural genes in a CreA derepressed strain, we propose a CreA-independent D-glucose inhibition mechanism that operates *via* XlnR. The presence of a CreA-independent mechanism of CCR that is involved in the regulation of transcription activators was suggested before. The isolation and analysis of constitutive mutants of the maltose-inducible regulator MAL63 from *S. cerevisiae* indicated that, in addition to transcription repression of the structural genes *via* MIG1, the CreA analogue, there is a D-glucose inhibiting mechanism independent of MIG1 (12). Also in *A. nidulans* a CreA-independent mechanism of CCR was proposed, based on comparison of expression levels of a reporter construct controlled by the *xlnB* promoter in a CreA wild type and CreA derepressed strain (21). A revised model for the regulation of the xylanolytic system is given in Figure 7. In the wild type strain (Fig. 7A), transcription of the structural genes is repressed *via* CreA (I) and *via* a second regulatory mechanism that holds XlnR in an inactive state (II). Upon induction, XlnR is converted into an active state and the structural genes are transcribed. When mutation

V756F is introduced in XlnR (Fig. 7B), the protein is permanently in an active state, whether or not inducer is present in the medium. In this state XlnR is insensitive to the regulatory mechanism that converts it into an inactive state in the presence of a repressing carbon source.

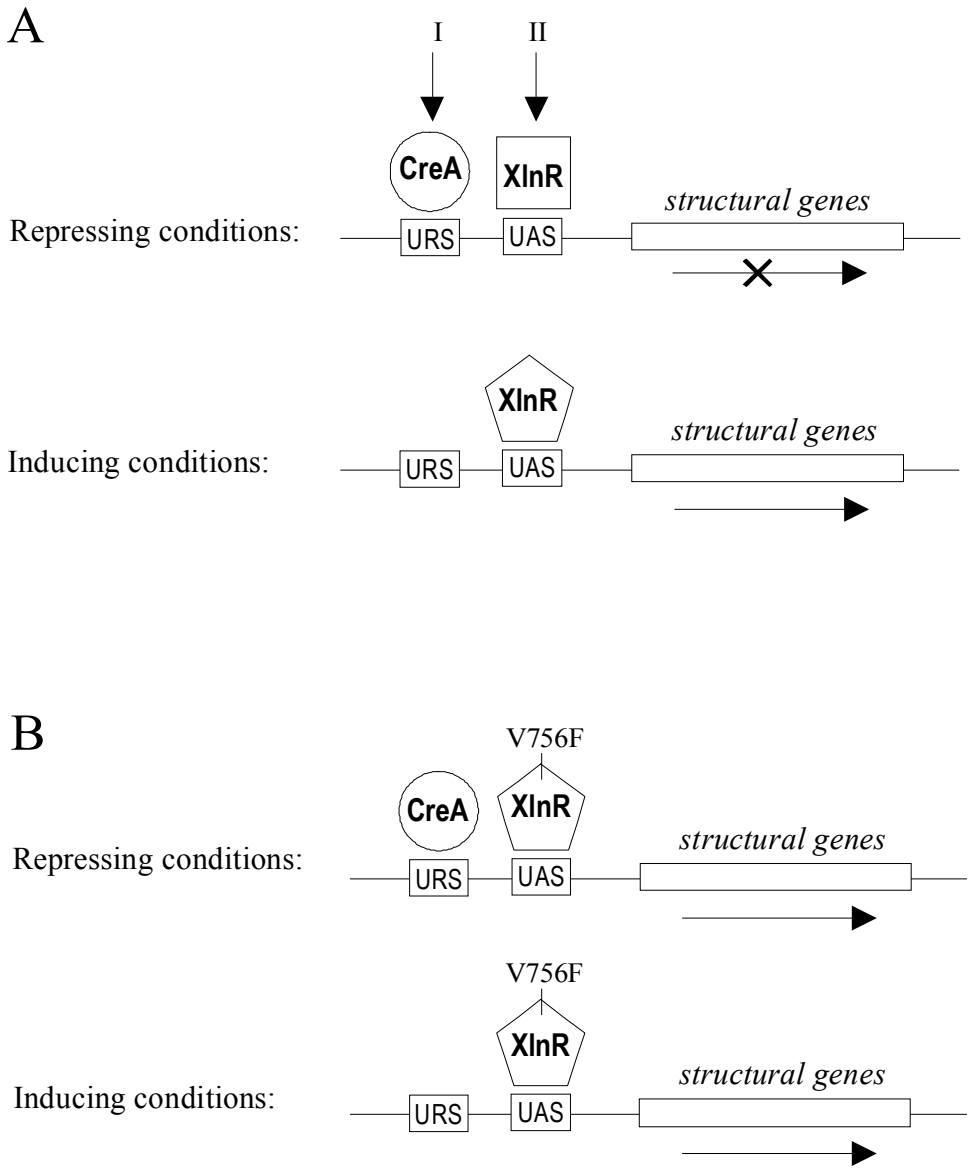

Fig. 7. New model for the regulation mechanism of XlnR controlled transcription. A. wild type strain; Under repressing conditions transcription of the structural genes is repressed *via* CreA. In the presence of a repressing carbon source XlnR remains in an inactive state. In the presence of inducer, XlnR becomes active resulting in transcription of its structural genes. B. Mutation V756F in XlnR brings XlnR in a permanent active state and makes XlnR insensitive to the D-glucose inhibition mechanism that transforms XlnR into an inactive state. This results in transcription of the structural genes under inducing as well as repressing conditions. The quadrangular form of XlnR represents an inactive protein; the pentagonal form represents the active protein.

Materials and methods

Strains, growth conditions and transformation procedure

All *A. niger* strains used were derived from N402, a low-conidiophore mutant from N400 (CBS 120.49) and are listed in Table 1. The media used were based on minimal medium (MM) (26) and were, when appropriate, supplemented with 1 mM L-arginine, 1.5 mM leucine, 2 mM lysine, 8 μM nicotinamide and/or 5 mM uridine. *A.niger*, inoculated at 10^6 spores ml^{-1}, was grown in shake flasks (250 rpm) at 30 ºC using a starting pH of 6. In transfer experiments, mycelia were precultured for 18 h in MM using 100 mM D-fructose as carbon source, supplemented with 0.1% yeast extract and 0.1 % casamino acids. The media used for induction contained 1% beechwood xylan (Sigma) or 50 mM D-xylose as inducing carbon sources. The mycelia were recovered and transferred as previously described (van Peij *et al*., 1998b). In transformation experiments the recipient strain was *A. niger* NW199 (*fwnA6, leuA5, goxC17, pyrA6, ΔxlnR::pIM240*) (10) or NW283 (*fwnA1, pyrA13, lysA7, creAd4*) (28). Transformation was carried out as described by Kusters-van Someren *et al*. (1991) (16). After transformation, a single copy integration of the mutated gene at the *pyrA* locus was selected through Southern blot analysis. For this a 3.8 kb *Bam*HI fragment containing the *pyrA* gene was cloned in pIM230 (23) resulting in pIM4444. Mutations in the *xlnR* gene were introduced in this plasmid *via* site-directed mutagenesis. For detection of endo-xylanase activity, transformants were plated onto MM plates containing 1% xylan or 50 mM D-glucose/1% xylan and 0.1% (v/w) azurin-dyed and cross-linked (AZCL) xylan (Megazyme, Wicklow, Ireland). After incubation at 30 ºC for 1 day, positive clones were identified by formation of blue haloes caused by the release of soluble dyed D-xylose oligomers from the insoluble substrate.

Genetic localisation and identification of the *xlnR^{c}5* mutation

Genes were localised on chromosomes using CHEF gel electrophoresis and Southern analysis as described by Verdoes *et al*. (1994) (32). The nature of the *xlnR^{c}5* mutation was analysed by cloning the *xlnR* gene, obtained from NW147 *via* PCR, in pGEMT, resulting in plasmid pR147A. Sequencing reactions were performed using the Thermo-Sequenase fluorescence-labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) using *xlnR*-specific Cy5 labelled oligonucleotides. Sequencing reactions were analyzed on an ALF*express* sequencer (Amersham Pharmacia Biotech).

Table 1. *A. niger* strains used in this work.

Strains	Genotype	Relevant phenotype[b]	Reference
NW219	*cspA1, nicA1, leuA1, pyrA6*	$XlnR^+/CreA^+$	
NW205::pIM130	*argB13, cspA1, nicA1, pyrA6, UAS(xlnA)-pyrA*	-	23
NW147	*pyrA6, xlnA-pyrA, xlnR^{c}5, nicA1, ArgB13*	$XlnR^c/CreA^+$	This work
NW199	*fwnA6, leuA5, goxC17, pyrA6, ΔxlnR:pIM240*	$XlnR^-/CreA^+$	10
NW199::pIM4449-24[a]	*pyrA6/pyrA$^+$, ΔxlnR:pIM240/xlnR$^+$:V756F*	$XlnR^c/CreA^+$	This work
NW199::pIM4466	*pyrA6/pyrA$^+$, ΔxlnR:pIM240/gfp::xlnR$^+$*	$XlnR^+/CreA^+$	This work
NW199::pIM4474	*pyrA6/pyrA$^+$, ΔxlnR:pIM240/pkiA(p)::gfp::xlnR$^+$*	$XlnR^+/CreA^+$	This work
NW283	*fwnA1, pyrA6, LysA7, CreA^{d}4*	$XlnR^+/CreA^d$	28
NW283::pIM4449-7[a]	*pyrA6/pyrA$^+$, CreA^{d}4, ΔxlnR:pIM240/xlnR$^+$:V756F*	$XlnR^c/CreA^d$	This work

[a] single copy integration in the *pyrA* locus

[b] $XlnR^+$ (XlnR wild type), $XlnR^-$ (*xlnR* knock-out), $XlnR^c$ (XlnR constitutive), $CreA^+$ (CreA wild type), $CreA^d$ (CreA derepressed)

General nucleic acid procedures

DNA manipulations were carried out using standard methods as described by Sambrook *et al*. (1989) (30). Fungal genomic DNA was isolated as previously described (8) and Southern blot analysis was performed according to standard methods (30) using the VacuGene XL Vacuum blotting system (Amersham Pharmacia Biotech). Total RNA was isolated from grinded mycelium using TRIzol reagent (Life Technologies, Rockville, MD). For northern blot analysis, RNA was separated in a 1.6 % agarose gel in 10 mM sodium phosphate buffer pH 7 and, after capillary blotting to Hybond-N (Amersham Pharmacia Biotech), hybridised in 50% formamide at 42 ºC. Filters were washed, once with 5*SSC, once with 2*SSC and twice with 0.2*SSC (1*SSC is 0.15 M NaCl plus 0.015 M sodium citrate) under homologous hybridisation conditions at 68 º C. The ^{32}P labelled DNA probes used were either cDNA or genomic fragments, as shown in Table 2.

Construction of Gfp-XlnR fusions

A codon-modified form of *gfp* (green fluorescent protein) that functions effectively in *A. nidulans* as a reporter for protein localisation was isolated from plasmid pMCB30 (5). The translation start codon of the *gfp* gene contains a *Nco*I site, while a *Nco*I site was introduced in the C-terminus of the coding region of the *gfp* gene in pMCB30. In the translation start codon of *xlnR* in pIM230 (23) a *Nco*I site was introduced. Then the *Nco*I fragment with the *gfp* gene was

cloned in this *Nco*I site in the appropriate orientation resulting in plasmid pIM4466. For construction of the *pkiA*-promoter fusion with *gfp-xlnR*, a 1 kb *Bam*HI-*Nco*I promoter fragment of pIM4466 was replaced by a 0.7 kb *pkiA* promoter fragment (8) resulting in plasmid pIM4474. In addition, the V765F mutation was introduced in XlnR in the latter construct, resulting in pIM4476.

Table 2. Probes used in northern blot analysis

Gene	EMBL	Enzyme encoded	Fragment used	Reference
faeA	Y09330	Feruloyl esterase A	0.5 kb *Eco*RV-*Xho*I [a]	33
xlnB	D38071	Endoxylanase B	0.9 kb *Eco*RI-*Xho*I	13
xlnD	Z84377	β-xylosidase D	2.8 kb *Pst*I-*Nsi*I [a]	22
xyrA	AF219625	D-xylose reductase	0.9 kb *Nco*I-*Xba*I	10
18S	X78538	18S rRNA subunit	0.7 kb *Eco*RI	19

[a] Genomic fragment instead of cDNA

Fluorescence microscopy

Samples of *A. niger* mycelia used for fluorescence microscopy were prepared by inoculating 200 µl of minimal medium containing 10 mM D-xylose and appropriate supplements with approximately 250 spores of *A.niger* transformants expressing GFP-XlnR fusion proteins. Inoculates were grown in chambered cover glasses (Nalge Nunc International Corp., Naperville IL.) for at least 24 h at 30 ºC. Samples of mycelia were assayed for fluorescence using the Zeiss Axiovert 100M microscope with the appropriate Zeiss filter combination (Excitation filter 470/40 dichoic 510 and LP 520). The nuclei in the mycelia were visualised using a 1:20 dilution DAPI-staining in Vecta-shield.

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CHAPTER 6

Functional analysis of the transcriptional activator XlnR from *Aspergillus niger*

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Abstract

The transcriptional activator XlnR from *A. niger* is a Zn binuclear cluster transcription factor that belongs to the GAL4 superfamily. Several putative structural domains in XlnR were predicted using database and protein sequence analysis. Thus far, only the functionality of the N-terminal DNA binding domain was determined experimentally.

We have constructed deletion mutants of the *xlnR* gene to localise the functional regions in the protein. Our results showed that a putative C-terminal coiled-coil region is involved in nuclear import of XlnR. After deletion of the C-terminus including this coiled-coil region, XlnR was found in the cytoplasm, while deletion of the C-terminus downstream of the coiled-coil region resulted in nuclear import of XlnR. The latter mutant also showed increased xylanase activity indicating the presence of a region having an inhibitory function in XlnR-controlled transcription. Previous findings already showed that a mutation in this C-terminal region in XlnR resulted in transcription of the structural genes under non-inducing conditions. A regulatory model of XlnR, in which the C-terminus responds to repressing signals that result in an inactive state of the protein, is presented.

Introduction

The transcriptional regulator XlnR from *Aspergillus niger* activates the transcription of genes encoding cellulose and hemicellulose degrading enzymes (van Peij *et al.*, 1998b, Gielkens *et al*., 1999, Hasper *et al*., 2000, Hasper *et al.*, 2002). XlnR belongs to the zinc binuclear cluster family of transcription factors exclusively found in fungi. Activation mechanisms of several zinc binuclear transcription factors have been described. An example of a well-characterised transcription regulator is GAL4 from *Saccharomyces cerevisiae*. GAL4 binds to the promoters of *gal* genes but is prevented from activating transcription by the inhibitory protein GAL80. Induction by D-galactose requires the action of the signal transducer protein GAL3. The GAL3-GAL80 interaction occurs in the cytoplasm and activates GAL4 in the nucleus (Blank *et al*., 1997, Peng and Hopper, 2002). Another mechanism of regulation is found for LEU3 from *S. cerevisiae*, the regulator of the leucine biosynthetic pathway. LEU3 undergoes intramolecular changes mediated by α-isopropylmalate (α-IPM) leading to unmasking of the activation domain and subsequent activation of transcription (Wang *et al*, 1997).

Only two regions of similarity between XlnR and other members of the GAL4 family have been found, the (Zn_2Cys_6) DNA-binding domain and amino acid motif Arg-Arg-Arg-Leu-Trp-Trp, which is a fungal specific transcription factor domain with unknown function (Suárez *et al*., 1995). Computer analysis of the XlnR amino acid sequence predicted the presence of a putative coiled-coil domain (Lupas *et al*., 1991) directly C-terminal to the DNA-binding domain and a second coiled-coil region at the C-terminal end of the protein. In a xylanase non-producing mutant, a single amino acid mutation was found in the latter coiled-coil region in XlnR (van Peij *et al*., 1998a). Two other loss-of-function mutations have been found in the C-terminal region of XlnR, which might indicate that this region contains the activation domain. In addition to these loss-of-function mutants, another mutant, which is constitutive in xylanase activity, has been isolated. Transcription studies showed that this mutation also affects the response of XlnR to D-glucose. This finding leads to the suggestion that the C-terminal region of the protein, in which this constitutive mutation has been found, is involved in modulation of XlnR activity. To gain insight into the mechanism by which XlnR regulates transcription of the target genes, several deletion mutants have been constructed based on the position of putative functional domains in XlnR. These mutants as well as XlnR mutants harbouring single amino acid mutations have been analysed for xylanase activity. Finally the cellular localisation of XlnR has been determined using a fluorescent Gfp-tag.

Results

Disturbance of C-terminal α-helix structure in XlnR results in an inactive XlnR

Molecular and functional analysis of XlnR regulatory mutants obtained *via* UV-mutagenesis, is a helpful tool in understanding structure-function relations within XlnR. Van Peij *et al*. (1998a) isolated several XlnR loss-of-function mutants to clone the *xlnR* gene *via* complementation. One of these mutants harbours an amino acid substitution, Leu650Pro, in the putative C-terminal coiled-coil region (Fig. 1a). Since introduction of a proline is known to disturb the helical structure of a coiled-coil domain, this mutation indicates that the coiled-coil structure is important for proper functioning of XlnR. To study this further, amino acid Leu650 has been substituted by isoleucine in plasmid pIM4444, harbouring the *xlnR* gene and a 3.8 kb fragment containing the *pyrA* gene (Hasper *et al*., 2003). The construct was used to transform NW199, a *xlnR* disruption mutant, and single copy integration was selected by Southern analysis. The xylanase activity of this mutant has been examined by AZCL-plate screening (Fig. 2). In the

Leu650Ile mutant xylanase activity decreased (Fig. 2b) compared to the wild type (Fig. 2a). It seems that a slight change in amino acid side-chain in this putative coiled-coil region is sufficient to result in decreased activity of XlnR. Database search for conserved amino acid motifs showed that in this putative coiled-coil region a potential tyrosine phosphorylation site (Patschinsky *et al*., 1982), KEFEARY, is found from amino acid position 658 to 664. A stop mutation within this potential motif, Tyr664stop, resulted in a XlnR loss-of-function mutant (Fig. 1b, 2d). To examine whether phosphorylation of Tyr664 plays a role in XlnR activity, the tyrosine has been replaced by a phenylalanine (Fig. 1a). This amino acid substitution does not allow phosphorylation at this position but maintains the secondary structure of the coiled-coil and the aromatic character of the residue at this position. In the Tyr664Phe mutant, the xylanase activity on D-xylose (Fig. 2c) as well as D-glucose (Fig. 2k) slightly decreased compared to the wild type (Fig. 2j), but did not result in loss of xylanase activity. Therefore this putative tyrosine phosphorylation site from amino acid position 658 to 664 is probably not involved in activation or inactivation of XlnR.

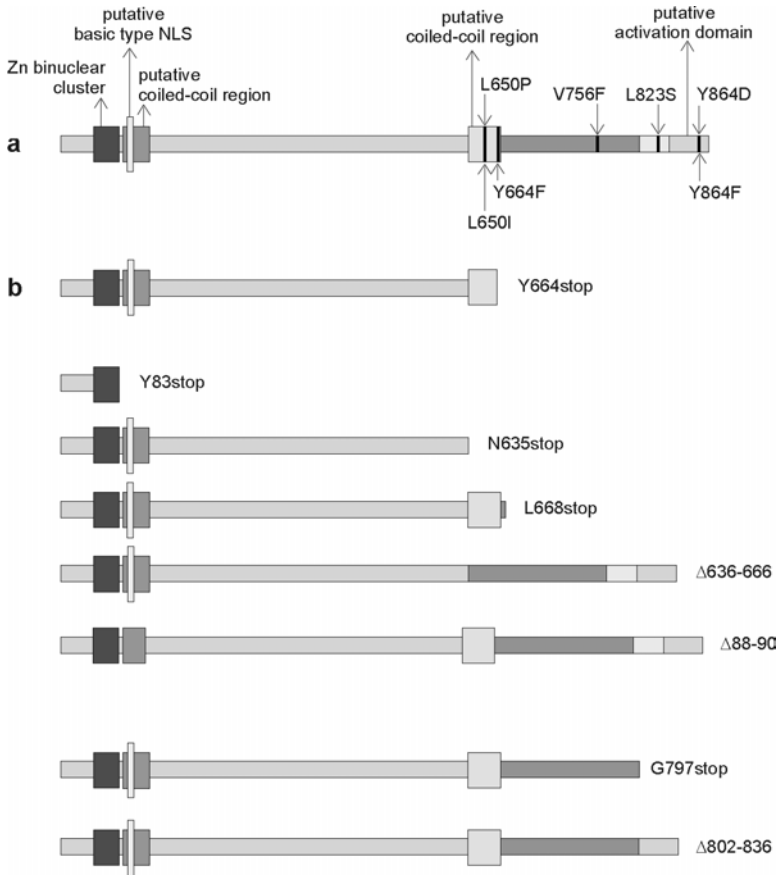

Fig. 1. Schematic presentation of the putative domain organization of XlnR; a. (putative) functional domains and amino acid substitutions; b. Deletion constructs of XlnR used in this study.

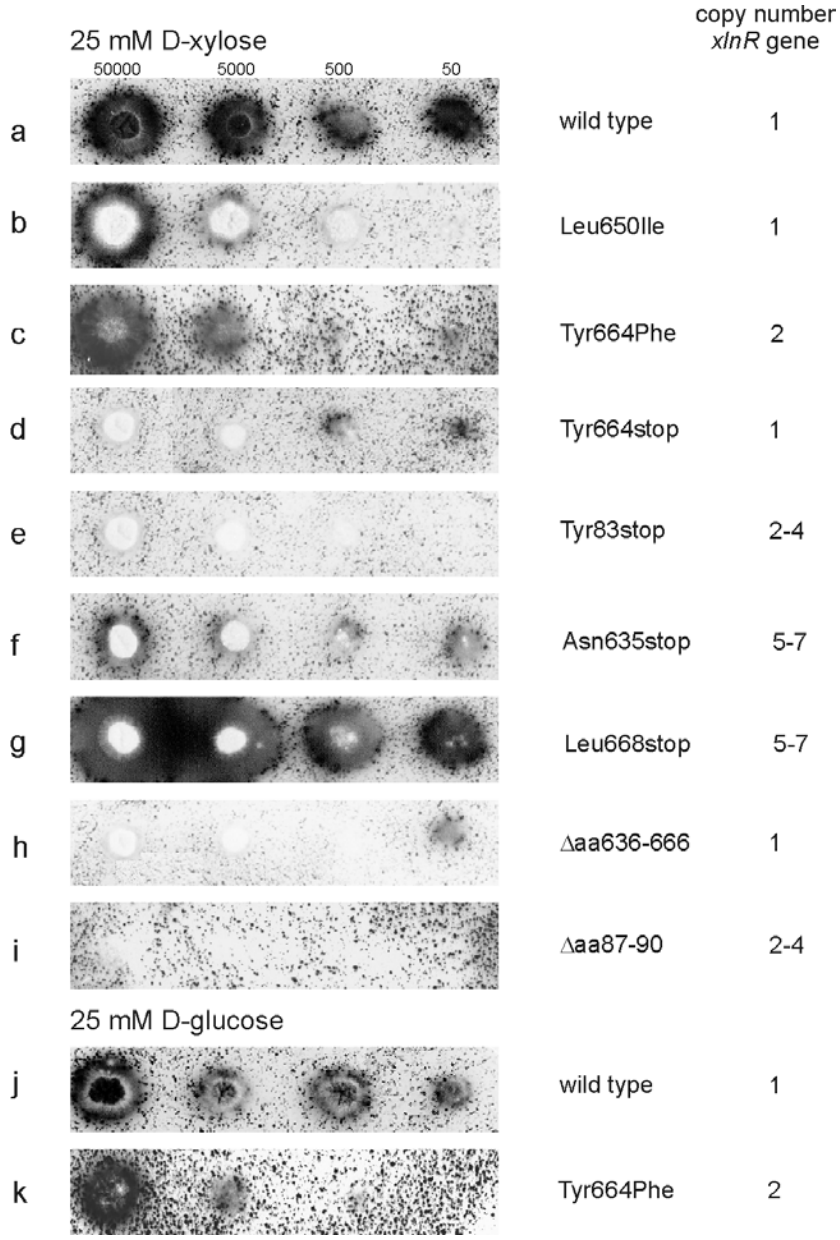

Fig. 2. AZCL plate screening of xylanase activity in *A. niger* wild type and *xlnR* mutants. Of each transformant, 50000, 5000, 500 and 50 spores were transferred to the AZCL xylan plates. Plates were incubated at 30 ºC for 2 days. The size of the blue haloes is indicative for the amount of xylanase activity produced by each transformant. In strains in which the *xlnR* gene is integrated in the *pyrA* locus, the copy numbers of the *xlnR* gene were determined *via* Southern blot analysis using a *pyrA* as a probe. In other strains (e, f and g), the copy number of the *xlnR* gene was determined by comparing the intensity of the fragments hybridised with the *xlnR* probe to an internal control.

The C-terminal putative coiled-coil domain in XlnR is involved in nuclear import

Based on the position of the putative functional domains in XlnR (Fig. 1a), deletion mutants (Fig. 1b) were made by introducing stop mutations in a *gfp-xlnR* fusion, driven by the *pkiA* promoter to obtain higher expression levels. Three stop mutations were introduced; Tyr83stop,

located in between the DNA-binding site and the putative basic cluster nuclear localisation signal (NLS); Asn635stop upstream, and Leu668stop downstream of the predicted C-terminal coiled-coil region. In order to create inducing conditions the mutants were grown on D-xylose and the cellular localisation of XlnR was determined. Confocal fluorescence microscopy demonstrated that XlnR deletion mutant Tyr83stop remains in the cytoplasm (Fig. 3b). In agreement with this, no xylanase activity was observed in this deletion mutant (Fig. 2e). In the wild type clear blue haloes resulting from xylan degradation are visible (Fig. 2a).

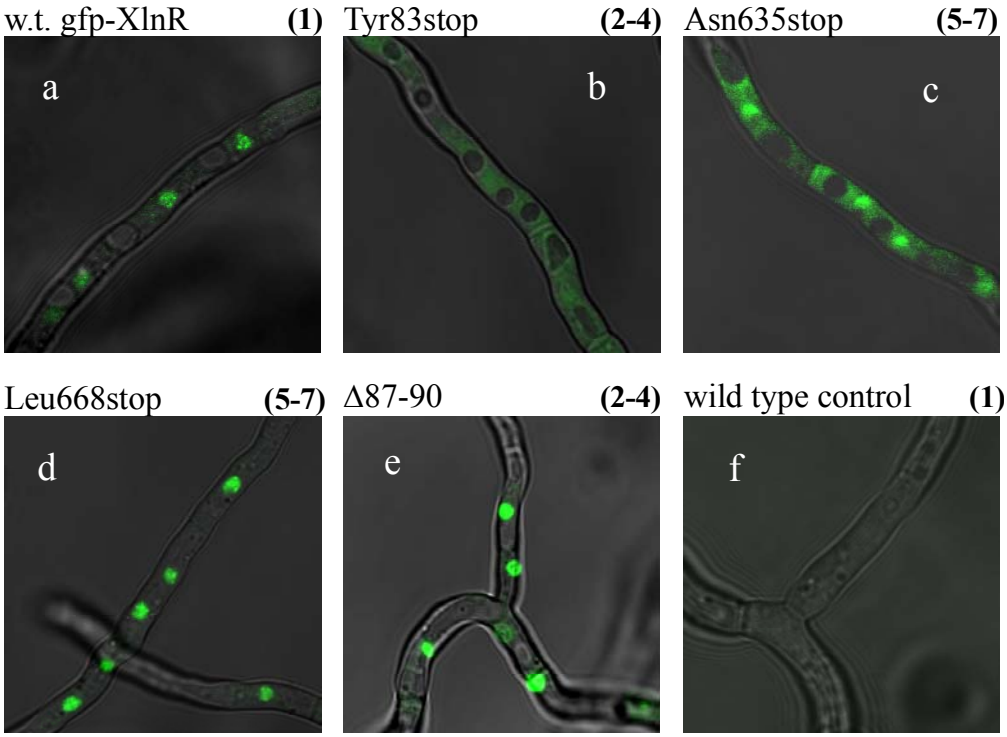

Fig. 3. Cellular localisation of wild type and mutated Gfp-XlnR fusion proteins driven by the *pkiA* promoter. a. Gfp-fusion with full-length wild type XlnR; b. Tyr83stop in XlnR; c. Asn635stop; d. Leu668stop in XlnR; e. Δ87-90 in XlnR; f. Control: wild type strain NW219. Between rounds, the copy number of the *xlnR* gene is (?) given. All strains were grown in cover glasses containing MM with 10 mM D-xylose for 24 h. Magnification is 160-200x.

Figure 3a shows the nuclear localisation of the wild type Gfp-XlnR fusion protein and figure 3f shows the non-fluorescent wild type as a control. After deletion of the C-terminal part including the putative C-terminal coiled-coil domain (Asp635stop), XlnR is also located in the cytoplasm although a minor part was found in the nucleus (Fig. 3c). The dark non-fluorescent circles in both mutants Tyr83stop and Asn635stop represent vacuoles. AZCL-xylan screening showed a low xylanase activity (Fig. 2f), which would be in agreement with the low amount of XlnR present in the nucleus. Deletion of the C-terminal part downstream of the putative coiled-coil domain (Leu668stop) resulted in nuclear localisation of XlnR (Fig. 3d) as in the wild type (Fig.

3a). The xylanase activity in this mutant (Leu668stop) is strongly increased (Fig. 2g) compared to the wild type. It appears that the first 667 amino acids are sufficient to give a fully functional XlnR and that the C-terminal coiled-coil region of XlnR is involved in nuclear import of the protein. The strongly increased xylanase activity in the Leu668stop mutant might be the result of the presence of multiple copies of the mutated *xlnR* gene that are integrated in the chromosome. Alternatively this effect might be explained by the theory that the C-terminus is involved in regulation of XlnR.

To confirm its role in nuclear import, amino acids 636 to 666 from XlnR that include the putative coiled-coil domain, were deleted. This resulted in total loss of xylanase activity (Fig. 2h) which is in agreement with the finding that XlnR is not transported to the nucleus. However, since no fluorescence has been observed determination of cellular localisation was not possible. Although there is only one copy of the mutated *gfp:xlnR* fusion gene present in the chromosome, the fact that it is driven by the constitutive *pkiA* promoter should result in increased levels of protein. Also no fluorescence has been observed in two other mutants harbouring a mutation located in the coiled-coil region, Leu650Pro and Tyr664stop. The first mutant contains multiple copies of the *gfp:xlnR* gene and the second mutant harbours a single copy integration of the gene driven by the *pkiA* promoter, at the *pyrA* locus. All these mutations resulted in xylanase non-producing mutants. It seems that a disturbed or deleted coiled-coil domain in a full-length protein affects the stability of the XlnR protein.

The classical monopartite basic cluster NLS in the N-terminus of XlnR is not functional

Most proteins are directed to the nucleus by a single classical type of NLS (Christophe *et al*., 2000). In the N-terminus of XlnR 4 basic clusters can be found, 1 and 2 upstream, and 3 and 4 downstream of the Zn2Cys6 DNA-binding domain (Fig. 4a). Neither cluster 1 and 2 nor 3 and 4 are conform to the bipartite NLS consensus sequence, $(K/R)_2$-$X_{10\text{-}12}$-$(K/R)_3$, of which the NLS of nucleoplasmin is the prototype (Nigg, 1997) (Fig. 4c). For transport of *Aspergillus nidulans* PrnA into the nucleus a tripartite nuclear localisation sequence is required (Pokarska *et al*., 2000). The three basic clusters that form the NLS in PrnA (1, 2 and 3) (Fig. 4b) are separated by only 5 and 8 amino acids. Since clusters 2 and 3 at the N-terminus of XlnR are separated by as many as 37 amino acids, it is not likely that the N-terminus of XlnR harbours a tripartite NLS as is found in PrnA. Based on the motifs of karyopherin α that recognise NLSs, Pokarska *et al*., (2000) suggested that there may be a relationship between the number of basic motifs comprising an NLS and the permitted distance between them. Cluster 3 that is formed by residues 87 to 90 is the only basic stretch that matches the consensus sequence of a monopartite

NLS, $(K/R)_{4\text{-}6}$, of which the NLS of the SV40 large T antigen is the prototype (Nigg, 1997). Since it has been shown that XlnR-mutant Asn635stop that lacks the C-terminal coiled-coil region, is partially located in the nucleus, there may be a second functional NLS in the N-terminus of XlnR: the basic cluster RKKR (Fig. 4a). An example of a protein that harbours two regions involved in nuclear import is the homeoprotein thyroid transcription factor (TTF-1). TTF-1 harbours both a classical monopartite NLS and a motif involved in nuclear import that is unrelated to known NLSs (Christophe-Hobertus *et al*., 1999).

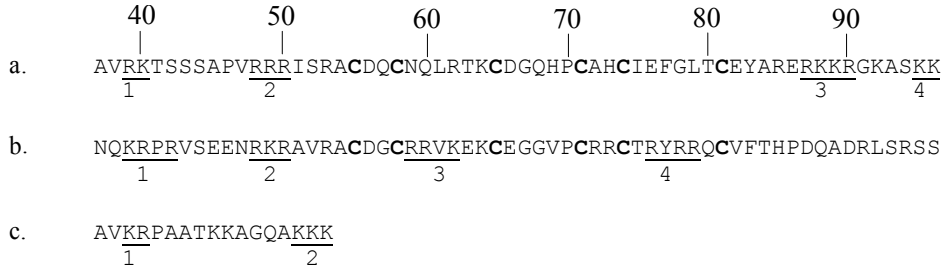

Fig. 4. Comparison of the basic clusters (underlined) found in the N-terminus of XlnR (a) to those of PrnA known to form a tripartite NLS (b) and those of nucleoplasmin forming a bipartite NLS (c) (Nigg, 1997). For XlnR the amino acid position is indicated with vertical lines.

To investigate the functionality of the monopartite NLS in XlnR, the two lysines in this cluster were deleted and one arginine has been replaced by a glycine *via* site-directed mutagenesis in the *pkiA:gfp:xlnR* construct (Δ88-90, Fig. 1b). The mutated gene was introduced in the Δ*xlnR* strain NW199 and cellular localisation has been determined using fluorescence microscopy. Figure 3e shows that partial deletion and mutation of the putative monopartial NLS in the N-terminus of XlnR does not affect nuclear localisation of the protein. Although the nuclear localisation of XlnR in this mutant was not affected, no xylanase activity has been found (Fig. 2i). Thus, although it appears that the basic cluster RKKR is not a functional NLS, the putative coiled-coil region in which it is located, seems to play an essential role in XlnR function. The low amount of the XlnR-mutant Asn635stop present in the nucleus may be due to another unidentified functional NLS within the first 635 amino acids of the protein that participates in nuclear localisation. Alternatively, the region involved in nuclear import might involve a part of the protein downstream of the putative coiled-coil domain. Thus, the precise regions or domains involved in nuclear localisation remain to be determined.

The C-terminal part downstream of the second coiled-coil domain of XlnR is involved in regulation.

Two xylanase non-producing mutants isolated by van Peij *et al*. (1998a) were shown to have single amino acid substitutions in the C-terminus of XlnR. This led to the suggestion that this part of the protein is involved in regulation of the activity of XlnR. In one of the mutants, the tyrosine at position 864 has been substituted by aspartate. Since phosphorylation is a general mechanism for responding to activating or inactivating signals, for example as in Pho4 from *S. cerevisiae*, (Komeili and O'shea, 1999), the tyrosine at position 864 has been replaced by a phenylalanine. The phenylalanine will not disturb the secondary structure of XlnR but can not be phosphorylated. This mutation resulted in strongly decreased xylanase activity on D-xylose while on D-glucose the activity was comparable to the wild type (Fig. 5a and c). This means that phosphorylation of this tyrosine might play a role in regulation of XlnR. The xylanase activity of the wild type grown on the D-glucose containing plates can be explained by the local consumption of D-glucose resulting in derepression and induction of the xylanolytic genes by AZCL-xylan. In the Δ*xlnR* strain, xylanase activity has been found surrounding the low spore-inoculates grown on D-xylose while this is not the case for the higher spore-inoculates. Determination of the xylanase activity *via* AZCL-plate screening is a semi-quantitative method; however, this can not explain the halo-formation observed at the low spore-inoculates of the Δ*xlnR* strain.

Recently it was found that a single amino acid mutation Val756Phe in XlnR resulted in xylanase activity under repressing conditions (Fig. 5d) (Hasper *et al*., 2003). Also a high xylanase activity was found under repressing conditions in a mutant harbouring a stop mutation at position Leu668 (Fig. 5e). The higher xylanase activity in the Leu668stop mutant compared to the Val756Phe mutant is probably due to the higher copy number of the *xlnR* gene in this mutant. These two mutants indicate that the C-terminal region of XlnR, downstream of the coiled-coil region, might be involved in modulation of the XlnR activity in the presence of D-glucose. The two loss-of-function mutations within the last 60 amino acids of XlnR also suggest this region is relevant for XlnR functioning. To examine this further, two C-terminal deletion mutants of XlnR were constructed (Fig. 1b), based on the position of the two loss-of-function mutations, Leu823Ser and Tyr864Asp (Fig. 1a). In one mutant, amino acids 802 to 836, encompassing Leu823, were deleted. In the second mutant, a C-terminal fragment of 78 amino acids has been removed by introducing a stop codon at position Gly797. Analysis of the xylanase activity on AZCL-plates with D-xylose showed that deletion of the C-terminal 78 amino acids resulted in an increased xylanase activity compared to the wild type (Fig. 5f). In

contrast, deletion of amino acid 802-836 resulted in complete loss of xylanase activity on D-xylose (Fig. 5g), as in Δ*xlnR* mutant NW199 (Fig. 5b). These different results could be explained by a regulation mechanism through inter- or intramolecular interactions within the C-terminus. Deletion of the C-terminal 78 amino acids might disable the mechanism, which results in inactivation, but will leave the secondary structure intact. Deletion of an intermediate part of this C-terminus might disturb the secondary structure in such a way that XlnR is turned into in an irreversible inactive state. This occurs for example in case the interaction of XlnR with the transcription machinery is obstructed. Thus, we hypothesise that the XlnR activity is regulated through inter- or intramolecular interaction in its C-terminal end.

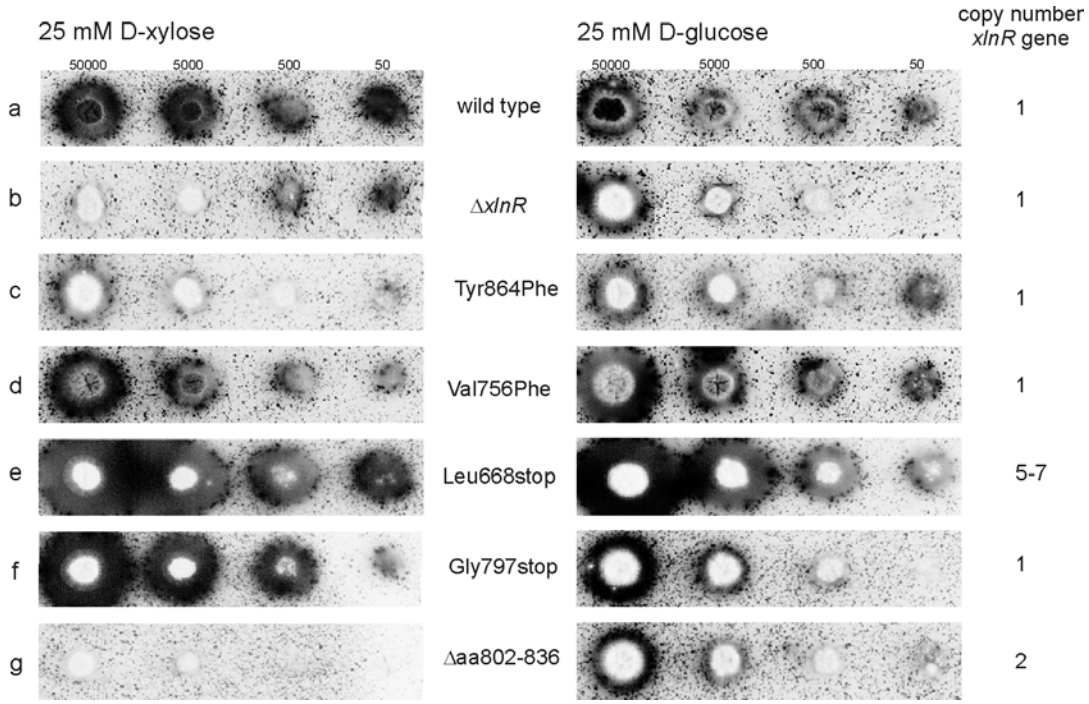

Fig. 5. AZCL plate screening of xylanase activity in *A. niger* wild type and *xlnR* mutants. Of each transformant, 50000, 5000, 500 and 50 spores were transferred to the AZCL xylan plates. Plates were incubated at 30 ºC for 2 days. The size of the blue haloes is indicative for the amount of xylanase activity produced by each transformant. In strains in which the *xlnR* gene is integrated in the *pyrA* locus, the copy numbers of the *xlnR* gene were determined *via* Southern blot analysis using a *pyrA* as a probe. Otherwise (e) the copy number of the *xlnR* gene was determined by comparing the intensity of the fragments hybridised with the *xlnR* probe to an internal control.

Discussion

The isolation of a loss-of-function mutation in the putative C-terminal coiled-coil domain of XlnR (van Peij *et al*., 1998a), already indicated the functional importance of this region. Additional mutation and deletion studies described in this paper show that maintaining the structure of this domain is essential for proper functioning of XlnR (Fig. 2b, c, d, and h). Coiled-coil regions were found in other transcriptional activators. They are often located near the N-terminus and are involved in dimerisation, such as in the NirA or PrnA transcription factors from *Aspergillus nidulans* (Strauss *et al*., 1998, Cazelle *et al*. 1998). In some transcription factors, such as PrnA, a second putative coiled-coil region is predicted in the C-terminal part of the protein. However, no function has been described for this region in XlnR. Cellular localisation of a XlnR mutant, in which the C-terminus including the coiled-coil region has been deleted, showed that this domain is involved in nuclear import of the protein. This suggests the presence of a nuclear targeting signal, although no classical NLS motif could be found in this region. Several sequence motifs were reported to mediate the nuclear import of proteins, besides the classical basic-type of NLS, which is the most common one. An example is the M9 sequence from hnRNP A1 and A2 proteins (Pollard *et al*., 1996). This sequence is recognised by transportin (Trn), a close homologue of importin-β, which alone or in a heterodimeric complex with importin-α, is responsible for the translocation of proteins harbouring classical NLSs through the nuclear pore (Truant *et al*., 1999, Palmeri *et al*., 1999). A consensus Trn interaction motif was determined to be (YFW)XXJXSXZG(PK)(MLV)(KR) (Bogerd *et al*., 1999). This motif could not be found in the XlnR putative coiled-coil region. Another example of a protein with an alternative NLS is the FAS-associated factor qFAF, a nuclear protein with an unknown function. Its nuclear targeting signal resides in a region that includes a α-helix (Fröhlich *et al*., 1998), which also appears to be the case for XlnR. Previously, a putative basic cluster NLS was identified in the N-terminus of XlnR. However, cellular localisation of a XlnR mutant in which this potential NLS is mutated, showed that it is not involved in nuclear localisation (Fig. 3e). The region, in which this basic cluster is located, is a potential coiled-coil domain. Since the XlnR-binding site was determined to bind to a non-palindromic consensus in the promoter, it was suggested that XlnR binds as a monomer (van Peij, *et al*. 1998a). Therefore, the N-terminal coiled-coil domain is not likely to be involved in dimerisation, even though it is closely linked to the DNA-binding domain. Nevertheless, the XlnR mutant in which the putative NLS has been mutated was not active. Apparently, this putative helical region is also important for XlnR functioning.

Several members of the Zn_2Cys_6 transcription factor family were shown to harbour a C-terminal regulatory domain. In GAL4, the activation domain resides between amino acids 768 to 881, the last 113 residues of the protein (Keegan *et al*., 1986, Ma and Ptasche, 1987). In LEU3 (886 amino acids), the activation domain is located within residue 861 to 886 and in PUT3 (979 amino acids), the activator of the proline utilisation pathway, the C-terminus from amino acid 890 to 979 is shown to include domains for activation and regulation (des Etages *et al*., 1996). Regulatory mutations were found within the last 120 amino acids of the XlnR C-terminus. Deletion of the complete C-terminus downstream of the predicted coiled-coil region leaves a fully active protein, as seen in XlnR mutant Tyr668stop. This indicates that this region more likely responds to repressing signals than to inducing signals. It is in agreement with the theory that mutation Val756Phe, resulting in xylanase expression under repressing conditions, disturbs a D-glucose inhibitory domain. Characterisation of two deletion mutants, one of which lacks an internal fragment of 34 amino acids (from residue 802-836) and another one of which the last 78 residues were deleted, led to a hypothetical model in which the C-terminus is involved in regulating the activity of XlnR. In this model, a proposed D-glucose inhibition domain in the C-terminus, downstream of Leu668, responds to repressing signals *via* intra- or intermolecular interactions which turn XlnR into an inactivated state. Our results lead to a new model for the mechanism by which XlnR is regulated and which is presented in Figure 6a.

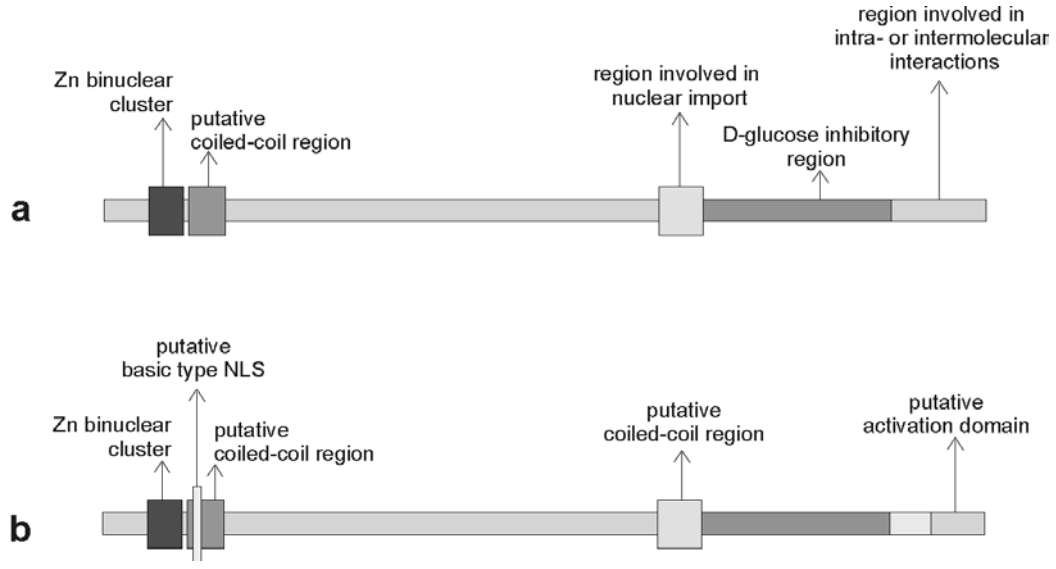

Fig. 6. In this paper complex data were used to determine structure-function relations in XlnR. To clarify these data, a new schematic presentation of the domain organisation in XlnR is proposed (a) and placed next to the previous model (b). In the new model the putative C-terminal coiled-coil region is involved in nuclear import, while the conserved basic cluster in the N-terminus is shown not to be a nuclear transport signal. The C-terminus downstream of the coiled-coil region fulfils a regulatory role in XlnR. Although not determined precisely, a part of this region harbours a D-glucose inhibitory domain. We propose that the C-terminus is involved in intra- or intermolecular interactions.

Methods

Strains, growth conditions and transformation procedure

All *A. niger* strains used were derived from N402, a low-conidiophore mutant from N400 (CBS 120.49) and are listed in Table 1. The media used were based on minimal medium (MM) (Pontecorvo *et al*., 1953) and were, if appropriate, supplemented with 1.5 mM leucine, 8 μM nicotinamide and/or 5 mM uridine. In transformation experiments the recipient strain was *A. niger* NW199 (*fwnA6, leuA5, goxC17, pyrA6, ΔxlnR::pIM240*). For transformation, *A. niger* was inoculated at 10^6 spores ml^{-1} and grown in shake flasks (250 rpm) at 30 ºC, using a starting pH of 6. Transformation has been carried out as described by Kusters-van Someren *et al*. (1991). After transformation, for some of the *xlnR* transformants a single copy integration of the construct containing the mutated gene at the *pyrA* locus was selected through Southern blot analysis.

Table 1. Strains used in this work

Strain	Genotype
NW219	*cspA1, nicA1, leuA1, pyrA6*
NW199	*fwnA6, leuA5, goxC17, pyrA6, ΔxlnR*:pIM240
NW199::pIM4445-6[a]	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*xlnR:*L650I
NW199::pIM4448	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*xlnR:*Y664F
NW199::pIM4449-24[a]	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*xlnR:*V756F
NW199::pIM4450-1[a]	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*xlnR:*Y864F
NW199::pIM4466	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*gfp::xlnR*
NW199::pIM4455	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*gfp::xlnR:*L650P
NW199::pIM4474	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*pkiA(p)::gfp::xlnR*
NW199::pIM4457-2	*pyrA6, Δ*xlnR*:*pIM240/*pkiA(p)::gfp::xlnR:*Y83stop
NW199::pIM4458-1	*pyrA6, Δ*xlnR*:*pIM240/*pkiA(p)::gfp::xlnR:*N635stop
NW199::pIM4459-3	*pyrA6, Δ*xlnR*:*pIM240/*pkiA(p)::gfp::xlnR:*L668stop
NW199::pIM4512-4[a]	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*pkiA(p):: gfp::xlnR:*Y664stop
NW199::pIM4514-13[a]	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*pkiA(p):: gfp::xlnR:*G797stop
NW199::pIM4511-4[a]	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*pkiA(p):: gfp::xlnR*:Δ636-666
NW199::pIM4513-5	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*pkiA(p)::xlnR*:Δ802-836
NW199::pIM4518-6	*pyrA6/pyrA*$^+$*, Δ*xlnR*:*pIM240/*pkiA(p)::xlnR*:Δ88-90

[a] single copy integrations in the pyrA locus

Mutations in the *xlnR* gene have been introduced *via* site-directed mutagenesis in plasmid pIM4444 that harbours a 3.8 kb *Xba*I-fragment containing the *pyrA* gene, or pIM4474 that harbours a *gfp:xlnR* fusion construct (Hasper *et al*., 2003) (Table 2). For analysis of endo-xylanase activity, transformants have been plated onto MM plates containing 25 mM D-xylose or 25 mM D-glucose, and 0.1% (v/w) azurin-dyed and cross-linked (AZCL) xylan (Megazyme, Wicklow, Ireland). Spore solutions of the selected transformants have been diluted in Saline-Tween (ST) to concentrations of 10000, 1000, 100 and 10 spores per µl. Of each dilution 5 µl has been transferred to the AZCL xylan plates. After incubation at 30 ºC for 2 days, blue haloes were formed by the release of soluble dyed xylan oligomers from the insoluble substrate.

Table 2. Plasmids used in this work

Plasmid	**Description**
pIM230[a]	5 kb *Bam*HI-*Xba*I *xlnR* library clone
pIM4444[c]	3.8 kb *Xba*I *pyrA* in pIM230
pIM4445	*xlnR*:L650I in pIM4444
pIM4448	*xlnR*:Y664F in pIM4444
pIM4449	*xlnR*:V756F in pIM4444
pIM4450	*xlnR*:Y864F in pIM4444
pIM4465	*gfp::xlnR* in pIM230
pIM4466[c]	pIM4465 + *pyrA*
pIM4455	*xlnR*:L650P in pIM4466
pIM4474[c]	*pkiA(p)::gfp::xlnR* in pIM230
pIM4457	*xlnR*:Y83stop in pIM4474
pIM4458	*xlnR*:N635stop in pIM4474
pIM4459	*xlnR*:L668stop in pIM4474
pIM4512	*xlnR*:Y664stop in pIM4474 + *pyrA* [b]
pIM4514	*xlnR*:G797stop in pIM4474 + *pyrA* [b]
pIM4511	*xlnR*:Δaa636-666 in pIM4474 + *pyrA* [b]
pIM4513	*xlnR*:Δaa802-836 in pIM4474 + *pyrA* [b]
pIM4518	*xlnR*:Δaa88-90 in pIM4474 + *pyrA* [b]

[a] van Peij *et al*., 1998

[b] A 3.8-kb *Xba*I-fragment was cloned into the multiple cloning site of the plasmid.

[c] Hasper *et al*., 2003

General DNA techniques

Standard genetic techniques were used according to Sambrook *et al*., (1989). Sequencing reactions were performed using the Thermo-Sequenase fluorescence-labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) using *xlnR*-specific Cy5 labelled oligonucleotides. Sequencing reactions were analysed on an ALF*express* sequencer (Amersham Pharmacia Biotech). Fungal genomic DNA was isolated as previously described (de Graaff *et al*., 1988) and Southern blot analysis was performed according to standard methods (Sambrook *et al*., 1989) using the VacuGene XL Vacuum blotting system (Amersham Pharmacia Biotech).

Fluorescence microscopy

Samples of *A. niger* mycelia used for fluorescence microscopy were prepared by inoculating 200 µl of minimal medium with 10 mM D-xylose and 10 mM D-glucose and appropriate supplements with approximately 250 spores of *A.niger* transformants expressing GFP-XlnR fusion proteins. Inoculates were grown in chambered cover glasses (Nalge Nunc International Corp., Naperville IL.) for 24 to 30 h at 30 °C. Samples of mycelia were assayed for green fluorescence using the Zeiss Axiovert 100M microscope with the appropriate Zeiss filter combination (Excitation filter 470/40 dichoic 510 and LP 520). The nuclei in the mycelia were visualised using a 1:20 dilution DAPI-staining in Vecta-shield.

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CHAPTER 7

General and Summarising Discussion

General and summarising discussion

Aspergillus niger degrades the polysaccharides from plant cell walls for consumption by secreting a wide variety of polysaccharide-degrading enzymes. Since plant cell walls can vary in their polysaccharide composition, the fungus regulates its enzyme production to efficiently utilise the carbon source that is available.

XlnR-controlled genes from *A. niger*

The transcriptional regulator XlnR controls the expression of a group of extracellular polysaccharide-degrading enzymes. In the presence of xylan, and the absence of a preferable carbon source such as D-glucose, XlnR activates the transcription of genes encoding xylanolytic and cellulolytic enzymes (Table 1) (van Peij *et al*. 1998b, Gielkens *et al*., 1999). It also activates the transcription of several accessory xylanolytic enzymes (Gielkens *et al*., 1997, de Graaff *et al*., 1992, de Vries *et al*., 1997, de Vries *et al*., 1998). This enables the fungus to completely degrade the cellulose and hemicelluloses such as arabinoxylan from the plant cell wall in a co-ordinate way.

To make an inventory of the spectrum of genes encoding enzymes involved in arabinoxylan and cellulose degradation, a differential cDNA library was constructed. This differential cDNA library of XlnR-controlled gene fragments was constructed by subtracting the cDNA of a D-xylose grown XlnR loss-of-function mutant from the cDNA of a D-xylose induced wild-type strain. Four new XlnR-controlled genes were isolated (Table 1, printed in bold), among which a gene encoding an intracellular enzyme (Chapter 2) and a gene encoding an endoxyloglucanase (Chapter 3). Also two genes encoding unknown enzymes were isolated. Based on its deduced amino acid sequence, one of these genes encodes for a transaldolase-like enzyme. The enzyme product of the other gene was, after purification, crystallisation and structure determination, shown to belong to the α/β-hydrolase fold family of proteins.

Table 1: XlnR-regulated genes from *A. niger*. Newly isolated genes described in this thesis are printed in bold.

Gene	Enzyme	Reference
cbhA	Cellobiohydrolase A	Gielkens *et al*., 1999
cbhB	Cellobiohydrolase B	Gielkens *et al*., 1999
eglA	Endoglucanase A	Van Peij *et al*., 1998b
eglB	Endoglucanase B	Van Peij *et al*., 1998b
eglC	**Endoglucanase C**	Hasper *et al*., 2002
aguA	α-Glucuronidase A	De Vries *et al*., 1998
axeA	Acetylxylan esterase	De Graaff *et al*., 1992
axhA	Arabinoxylan hydrolase A	Gielkens *et al*., 1997
faeA	Feruloyl esterase A	De Vries *et al*., 1997
xlnB	Endoxylanase B	Kinoshita *et al*., 1995
xlnC	Endoxylanase C	Gielkens *et al*., 1998
xlnD	β-Xylosidase	Van Peij *et al*., 1997
xyrA	**D-xylose reductase**	Hasper *et al*., 2000
talB	**Transaldolase B**	This thesis
estA	**Esterase A**	Bourne *et al*., in prep.

XlnR-controlled genes encoding intracellular enzymes

To metabolise the sugars of the plant cell wall, extracellular enzymes are required to degrade the polysaccharide into smaller molecules. The resulting mixture of monomeric sugars can be taken up by the fungus and used for metabolic processes. In case of xylan degradation, D-xylose is the major type of monomeric sugar that is released. To metabolise this sugar efficiently after uptake, the fungus needs to adjust its intracellular metabolic process (Fig. 1).

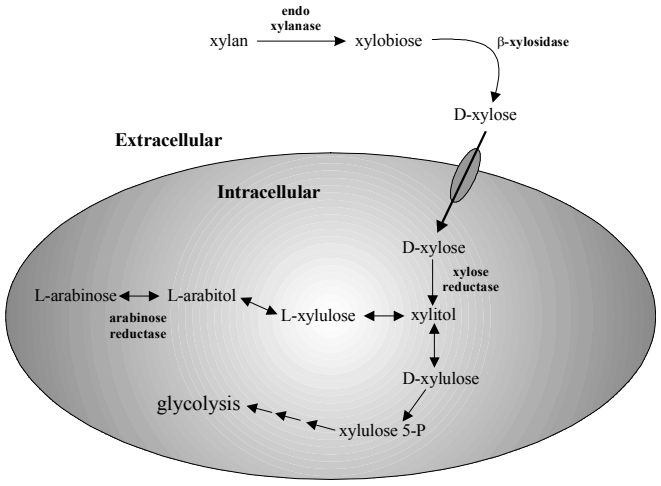

Fig. 1. Extracellular xylan degradation and intracellular D-xylose catabolism in *A.niger.*

The* xyrA *gene

Chapter 2 describes the isolation of the gene *xyrA*, encoding D-xylose reductase. D-xylose reductase catalyses the NADPH-dependent reduction of D-xylose to xylitol, which is the first step in D-xylose catabolism in fungi (Fig. 1). The isolation of this XlnR-regulated gene showed that XlnR not only regulates the transcription of genes encoding extracellular enzymes. By controlling the expression of *xyrA* by the same regulator, the fungus simultaneously adapts its intracellular sugar metabolism to the extracellular degradation of the hemicellulose xylan.

The functionality of *xyrA* was confirmed by disrupting the gene. In media with D-xylose as a sole carbon source there was no significant reduction of growth observed in plate assays of this strain, compared to the wild type. However, the D-xylose reductase activity in this *xyrA*-disrupted strain decreased with about 80 %. This inconsistency can be explained by the presence of L-arabinose reductase (see Fig. 1), which also has D-xylose reductase activity that apparently is sufficient for growth on D-xylose (Witteveen *et al*., 1989). It seems that by regulating the *xyrA* gene *via* XlnR, the fungus optimises its intracellular metabolism to process the increased levels of D-xylose that are released and taken up. Although transcription of *xyrA* is activated by XlnR, it is, unlike the other XlnR-controlled genes, not repressed by CreA. Considering the fact that intracellular D-xylose processing occurs in response to extracellular xylan degradation, CreA regulation at this point would be unnecessary. Since CreA regulates the genes encoding the extracellular enzymes involved in xylan degradation, repression of these genes will result in decreased xylanolytic activity and thus a decrease in D-xylose release. D-xylose reductase is required to process the D-xylose already taken up by the cell. Consumption of the D-xylose will eventually result in inactivation of XlnR and subsequently in reduced *xyrA* expression.

<u>*D-xylose uptake*</u>

Multiple copies of the *xyrA* gene in the *A. niger* genome led to a decrease in transcription of two other XlnR-controlled genes *xlnB* and *xlnD*. By placing the *xyrA* gene under control of the *pkiA* promoter, which is not regulated by XlnR, it was shown that the decreased expression of *xlnB* and *xlnD* is the result of titration of XlnR by the *xyrA* promoter and not the result of a decreased inducer concentration. A high *xyrA* copy number (15-20 copies), also resulted in a decrease in D-xylose consumption compared to the *A. niger* wild type strain. The same decrease in D-xylose uptake was found in a *xlnR*-disrupted strain. This effect could be explained by the presence of a XlnR-controlled D-xylose transporter. Since there is promoter titration of XlnR in the *xyrA* multiple copy strain, there is also a decreased transcription of this hypothetical gene, which results in decreased D-xylose uptake.

Likewise, disruption of *xlnR* would result in decreased expression of this gene. The fact that this strain is still capable of growth on D-xylose as a sole carbon source is probably due to the presence of low-affinity hexose transporters, which also transport D-xylose. For example in *S. cerevisiae*, D-xylose is transported into the cell *via* hexose transporters, which have a high affinity towards D-glucose ($K_m \approx 1$ mM) compared to D-xylose ($K_m \approx 200$ mM) (Kotyk, 1967). This type of transport is non-specific but results from the similarity in chemical structure of D-glucose and D-xylose. Since *S. cerevisiae* only utilises D-xylose in the presence of other substrates such as D-ribose, it does not need high affinity D-xylose transporters (Van Zyl *et al*., 1989). Yeast species like *Pichia heedii* and *Pichia stipitis* that utilise D-xylose more efficiently, were shown to have transport systems with high affinity for D-xylose ($K_m \approx 0.1$ mM and 0.9 mM resp.). Competition experiments indicated that these high affinity D-xylose transporters do not transport D-glucose but are regulated by D-glucose (Does and Bisson, 1990). Also the yeast *Debaryomyces hansenii* was shown to have two distinct high-affinity transport systems for D-glucose and D-xylose. In addition to the high-affinity D-xylose transport system, D-xylose also enters the cell *via* D-glucose-facilitated diffusion in this yeast species (Nobre *et al*., 1999). This facilitated diffusion system allows D-xylose to enter the cell and induce D-xylose high-affinity transport as well as the D-xylose catabolism-specific enzymes. A comparable system including a XlnR-regulated high-affinity D-xylose transporter could support D-xylose transport in *A. niger*.

In search for D-xylose permeases, a cDNA bank of *Trichoderma reesei* was screened for enhanced growth on D-xylose (Merja Penttilä. VTT Biotechnology and Food Research, Espoo, Finland, http://www.vtt.fi/bel/coe/patheng.htm). This resulted in the isolation of one permease clone, which supports growth of the D-xylose-utilising hexose transporter mutant yeast strain on D-xylose (but not on D-glucose), after a long lag phase of several weeks. When the strain is cultivated for a second time on D-xylose, the lag phase is considerably shorter. The presence of a D-xylose inducible transcriptional regulator such as XlnR could be responsible for this adaptation process.

Multiple sequence alignments of yeast sugar transporters and bacterial D-xylose proton symporters were used to identify regions of homology. Based on these homologous regions, primers were designed with the purpose to isolate a gene fragment from *A. niger* chromosomal DNA encoding a XlnR-controlled D-xylose transporter gene. Several fragments of genes with significant homology to different sugar transporters were isolated using PCR, however, Northern blot analysis showed that none of them was controlled by XlnR. The use of micro-array analysis could make the search for a XlnR-regulated D-xylose transporter gene more successful (Ekins and Chu, 1999, Kodama *et al*., 2002).

The talB *gene*

In D-xylose-fermenting yeasts and fungi, D-xylose is first reduced to xylitol, which is then oxidised to xylulose (Fig. 2). Xylulose is phosphorylated to xylulose 5-P and channelled *via* the pentose phosphate pathway (PPP) into glycolysis (Skoog and Hahn-Hagerdal, 1990). Transaldolase (EC 2.2.1.2.) controls, together with transketolase (EC 2.2.1.1), the metabolic flux in the nonoxidative PPP by catalysing the transfer of a dihydroxyaceton in the reversible reaction; sedoheptulose 7-P + glyceraldehyde 3-P ↔ fructose 6-P + erythrose 4-P (Fig. 2) (Wood, 1972, Wang *et al*., 1980).

To demonstrate the presence of a transaldolase orthologue in *A. niger*, degenerate primers were designed based on DNA sequences obtained from the *A. nidulans* genome database. This resulted in the isolation of a fragment from the *A. niger* genome encoding an enzyme that showed homology to enzymes from different organisms, *e.g.* Tal1 from *S. cerevisiae* and Tal1 from *Homo sapiens* (Fig. 3A) (Schaaff *et al*., 1990, Banki *et al*., 1994). The gene corresponding to the fragment was named *talA*, and is, considering its significant homology, likely to encode the transaldolase that fulfils a metabolic flux-controlling role in the PPP (Wood, 1972).

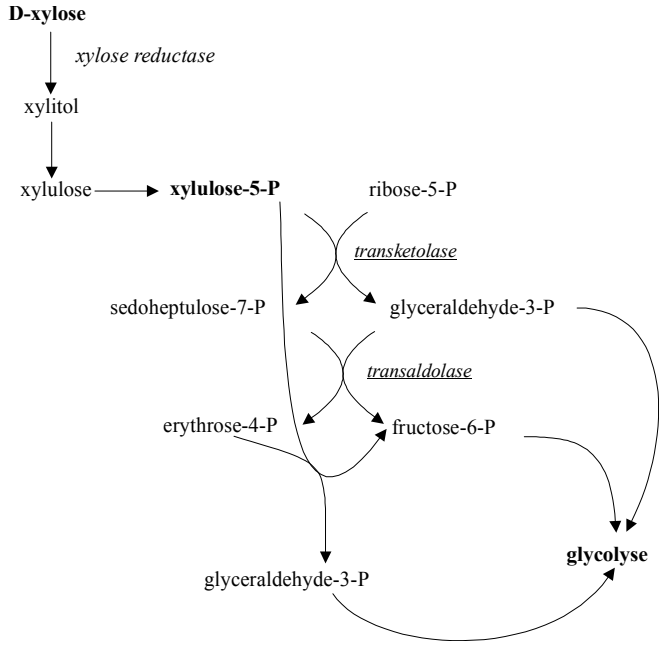

Fig. 2: Role of transaldolase (and transketolase) in the Pentose Phosphate Pathway

A typical transaldolase contains two consensus motifs, one located in the N-terminus (T-[ST]-N-P-[STA]-[LIVM]), and a second one that contains a highly preserved lysine residue [LIVM]-x-

[LIVM]-**K**-[LIVM]-[PAS]-x-T-x-[DENQ]-G-[LIVM]-x-A (Fig. 3). The lysine residue in this motif was shown to be the active site of translaldolases (Horecker *et al*., 1972, Miosga *et al*. 1993) and characteristic for Class I aldolases (Gefflaut *et al*., 1995). Figure 3A shows that the second conserved motif (double underlining) including the preserved lysine residue (in bold) can be found in the *talA* gene-fragment that was isolated. However, the complete gene encoding TalA needs to be cloned from *A. niger* to be able to confirm its functionality.

A.

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A.niger-TalA            ------------------------------------------------------------
S.cerevisiae-Tal1       MSEPAQKKQKVANNSLEQLKASGTVVVADTGDFGSIAKFQPQDSTTNPSLILAAAKQPTY 59
K.lactis-Tal1           MSEPSAKKQKFAN-SLEALKATGTTVVADTGDFESIAKFTPQDATTNPSLILAAAKQQAY 59
H.sapiens-Tal1          -MSSSPVKRQRMESALDQLKQF-TTVVADTGDFHAIDEYKPQDATTNPSLILAAAQMPAY 58
E.coli-TalA             ------------MNELDGIKQF-TTVVADSGDIESIRHYHPQDATTNPSLLLKAAGLSQY 47

A.niger-TalA            ---------------------------------------------VSTEVDARFSFDTQAS 16
S.cerevisiae-Tal1       AKLIDVAVEYGKKHGKTTEEQVENAVDRLLVEFGKEILKIVPGRVSTEVDARLSFDTQAT 119
K.lactis-Tal1           AKLIDSAVQYGKKQGQNIDEQVEIAVDKLLVEFGTAILKVVPGRVSTEVDARLSFDKDAT 119
H.sapiens-Tal1          QELVEEAIAYGRKLGGSQEDQIKNAIDKLFVLFGAEILKKIPGRVSTEVDARLSFDKDAM 118
E.coli-TalA             EHLIDDAIAWGKKNGKTQEQQVVAACDKLAVNFGAEILKIVPGRVSTEVDARLSFDKEKS 107
                                                            ********:*:*.:

A.niger-TalA            IDKALHIIKLYEENGISRDRILIKIASTWEGIKAAQVLQSQHGINCNLTLMFSTVQAIAA 76
S.cerevisiae-Tal1       IEKARHIIKLFEQEGVSKERVLIKIASTWEGIQAAKELEEKDGIHCNLTLLFSFVQAVAC 179
K.lactis-Tal1           VKKALEIIKLYEAEGISKDRVLIKIASTWEGIQAAQELEKEHDIHVNLTLLFSFAQAVAA 179
H.sapiens-Tal1          VARARRLIELYKEAGISKDRILIKLSSTWEGIQAGKELEEQHGIHCNMTLLFSFAQAVAC 178
E.coli-TalA             IEKARHLVDLYQQQGVEKSRILIKLASTWEGIRAAEELE-KEGINCNLTLLFSFAQARAC 166
                        : :* .::  :      :  :***::******:*.. *: :..*: *:**:** .** *.

A.niger-TalA            AEAGAYLISPFVGRILDWYKAAH-KRDYTAEEDPGVKSVQNIFNYYKKHGYKTIVMGASF 135
S.cerevisiae-Tal1       AEAQVTLISPFVGRILDWYKSST-GKDYKGEADPGVISVKKIYNYYKKYGYKTIVMGASF 238
K.lactis-Tal1           AEANVTLISPFVGRILDWYKAST-GETYTAETDPGVISVKSIYNYYKKHGYNTIVMGASF 238
H.sapiens-Tal1          AEAGVTLISPFVGRILDWHVANTDKKSYEPLEDPGVKSVTKIYNYYKKFSYKTIVMGASF 238
E.coli-TalA             AEAGVFLISPFVGRIYDWYQARKPMDPYVVEEDPGVKSVRNIYDYYKQHHYETIVMGASF 226
                        *** . ********* **: :      :    **** ** .*::***:. *:********

A.niger-TalA            RN------------------------------------------------------------ 137
S.cerevisiae-Tal1       RSTDEIKNLAGVDYLTISPALLDKLMNSTEPFPRVLDPVSAKKEAGDKISYISDESKFRF 298
K.lactis-Tal1           RNVGEIKALAGVDFLTISPKLLDELLSSDEPVAKILDPESAKAEGSERVSFINDEPKFRF 298
H.sapiens-Tal1          RNTGEIKALAGCDFLTISPKLLGELLQDNAKLVPVLSAKAAQASDLEKIH--LDEKSFRW 296
E.coli-TalA             RRTEQILALTGCDRLTIAPNLLKELQEKVSPVVRKLIPPSQTFPRPAPMS----EAEFRW 282
                        *

A.niger-TalA            ------------------------------------------
S.cerevisiae-Tal1       DLNEDAMATEKLSEGIRKFSADIVTLFDLIEKKVTA----- 334
K.lactis-Tal1           ELNEDAMATEKLSEGIRKFSADIVTLFDLIKAKIQA----- 334
H.sapiens-Tal1          LHNEDQMAVEKLSDGIRKFAADAVKLERMLTERMFNAENGK 337
E.coli-TalA             EHNQDAMAVEKLSEGIRLFAVDQRKLEDLLAAKL------- 316
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B.

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A.niger-TalB            ---------MTIKSGLDYIRSR-SIVDCDTMDEQVAKTFGPFHDCTSNQAIAYNELIKPE 50
S.cerevisiae-Tal1       MSEPAQKKQKVANNSLEQLKASGTVVVADTGDFGSIAKFQP-QDSTTNPSLILAAAKQPT 59
K.lactis-Tal1           MSEPSAKKQKFAN-SLEALKATGTTVVADTGDFESIAKFTP-QDATTNPSLILAAAKQQA 58
E.coli-TalA             -----------MN-ELDGIKQF-TTVVADSGDIESIRHYHP-QDATTNPSLLLKAAGLSQ 46
H.sapiens-Tal1          MSSSPVKRQRMES-ALDQLKQF-TTVVADTGDFHAIDEYKP-QDATTNPSLILAAAQMPA 57
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A.niger-TalB            HDELHTPLTQAKALIQEYPGLTIEELAIEIAMISLSLRIAPDITGHIHIQTNPYYSYSTE 110
S.cerevisiae-Tal1       YAKLIDVAVEYGKKHGKTT-EEQVENAVDRLLVEFGKEILKIVPGRVSTEVDARLSFDTQ 118
K.lactis-Tal1           YAKLIDSAVQYGKKQGQNI-DEQVEIAVDKLLVEFGTAILKVVPGRVSTEVDARLSFDKD 117
E.coli-TalA             YEHLIDDAIAWGKKNGKTQ-EQQVVAACDKLAVNFGAEILKIVPGRVSTEVDARLSFDKE 105
H.sapiens-Tal1          YQELVEEAIAYGRKLGGSQ-EDQIKNAIDKLFVLFGAEILKKIPGRVSTEVDARLSFDKD 116
                        : .*                     * :   : :.  *   :.*::  :.:.  *:..:

A.niger-TalB            KTIVNALRIVKLTHRLSPTHPQSHICIKIPSTWEGLKACQVLEQA-GVRTLATTLFTMPQ 169
S.cerevisiae-Tal1       ATIEKARHIIKLFE--QEGVSKERVLIKIASTWEGIQAAKELEEKDGIHCNLTLLFSFVQ 176
K.lactis-Tal1           ATVKKALEIIKLYE--AEGISKDRVLIKIASTWEGIQAAQELEKEHDIHVNLTLLFSFAQ 175
E.coli-TalA             KSIEKARHLVDLYQ--QQGVEKSRILIKLASTWEGIRAAEELEKE-GINCNLTLLFSFAQ 162
H.sapiens-Tal1          AMVARARRLIELYK--EAGISKDRILIKLSSTWEGIQAGKELEEQHGIHCNMTLLFSFAQ 174
                          : .* .::.* .       :.:: **:.*****::* : **:  .:.   * **:: *

A.niger-TalB            AILAAEVGCTYIAPYVNHSESPFSSQGN-------PSPRPHFSALAQKYYASIGSQTQVL 222
S.cerevisiae-Tal1       AVACAEAQVTLISPFVGRILDWYKSSTG-KDYKGEADPGVISVKKMYNYYKKYGYKTIVM 235
K.lactis-Tal1           AVAAAEANVTLISPFVGRILDWYKASTG-ETYTAETDPGVISVKSIYNYYKKHGYNTIVM 234
E.coli-TalA             ARACAEAGVFLISPFVGRIYDWYQARKPMDPYVVEEDPGVKSVRNIYDYYKQHHYETIVM 222
H.sapiens-Tal1          AVACAEAGVTLISPFVGRILDWHVANTDKKSYEP-LEPGVKSVTKIYNYYKKFSYKTIVM 233
                        *  .**.    *:*:*.:  . . :    .    . .*         .** .   :* *:

A.niger-TalB            PASLTSTNEIFALAGVQHITIAPGLLEELPSRWRGRYLWSRCLIPLLWWRGCAGKEVSYV 282
S.cerevisiae-Tal1       GASFRSTDEIKNLAGVDYLTISPALLDKLMNSTE---PFPRVLDPVSA-KKEAGDKISYI 291
K.lactis-Tal1           GASFRNVGEIKALAGVDFLTISPKLLDELLSSDE---PVAKILDPESA-KAEGSERVSFI 290
E.coli-TalA             GASFRRTEQILALTGCDRLTIAPNLLKELQEKVS---PVVRKLIPPSQ-TFP--RPAPMS 276
H.sapiens-Tal1          GASFRNTGEIKALAGCDFLTISPKLLGELLQDNA---KLVPVLSAKAA-QASDLEKIHLD 289
                         **:  . :*  *:* : :**:* ** :* .           * .

A.niger-TalB            NDESGYRIAFTRDAGGLSEEKLTQ-VRYYCDMQDKLVQVFKGLN------- 325
S.cerevisiae-Tal1       SDESKFRFDLNEDA--MATEKLSEGIRKFSADIVTLFDLIEKKVTA----- 335
K.lactis-Tal1           NDEPKFRFELNEDA--MATEKLSEGIRKFSADIVTLFDLIKAKIQA----- 334
E.coli-TalA             --EAEFRWEHNQDA--MAVEKLSEGIRLFAVDQRKLEDLLAAKL------- 316
H.sapiens-Tal1          --EKSFRWLHNEDQ--MAVEKLSDGIRKFAADAVKLERMLTERMFNAENGK 336
                        ..*  :*   ..*   :: ***::.:* :.    .*  ::
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Fig. 3: Amino acid sequence alignment of TalA (A) and TalB (B) with transaldolases from several other organisms. The amino acid conservation between the transaldolases from (A) is about 50 to 60 %, the conservation of these transaldolases withTalB is about 30 %. Numbers indicate amino acid position. Dashes indicate a gap. Asterix indicate an identical residue in all five proteins, whereas dots represent conservative changes. The N-terminal conserved motif is underlined and a double underlining marks the second consensus motif. In this second motif the highly preserved active site lysine is shown in bold. The comparison was done using the program CLUSTAL W (http://www.ebi.ac.uk/clustalw/). The amino acid sequences from the different transaldolases are obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein).

A new gene encoding an intracellular enzyme with homology towards transaldolases was isolated. This putative enzyme consists of 330 amino acids and showed about 30 % homology with the highly conserved transaldolases found in other organisms (Fig 3B). Since there is evidence for the presence of a transaldolase (EC 2.2.1.2) orthologue in *A. niger*, this new transaldolase-like enzyme was named TalB. From the two consensus motifs that are preserved in transaldolases, the first one is for a major part present (T-S-N-Q-A-I) in the N-terminus of TalB, at amino acid positions 36 to 41. A full match to the second consensus motif can be found from amino acid positions 136 to 149. Therefore it is expected that TalB is a new ClassI transaldolase-like enzyme in *A. niger*. This family of enzymes is not related in amino acid

sequence and catalyses different types of aldol cleavage/condensation reactions (Gefflaut *et al*., 1995). Based on the fact that XlnR controls the *talB* gene, the enzyme might fulfil a supportive role in processing the intracellular levels of D-xylose. It might for instance provide a short-cut for the introduction of D-xylose into glycolysis. To determine the role of TalB in intracellular D-xylose metabolism, a *talB* disruption strain and *talB* multiple copy strain were constructed and after growth on D-xylose, the level of several intracellular metabolites, such as xylulose-5-P, fructose-6-P and ribose-5-P, was measured in both strains. No significant difference in intracellular metabolite concentration was noticed. However, experiments have to be repeated and optimised to be able to draw legitimate conclusions. The TalB protein was purified using an N-terminal His-tag. To examine its function the purified TalB proteins can be used in (trans-) aldolase reactions with pentose or pentitole sugars. Since xylulose 5-P is the intermediate that enters the PPP, this sugar might be an interesting substrate.

EglC, a new endoglucanase

The isolation of a XlnR-controlled gene encoding a new endoglucanase, EglC, is described in Chapter 3. It fulfils a unique function in the polysaccharide degradation process since it is active on xyloglucan. This third endoglucanase from *A. niger* consists of 858 amino acids and contains a conserved C-terminal cellulose-binding domain (CDB). In *Aspergillus aculeatus*, the enzyme AviIII, which has 75 % homology to EglC, was found (Takada *et al*., 1998). This enzyme is described as an exoglucanase while EglC is clearly an endoglucanase (Fig. 2, Chapter 3). In *Trichoderma viride* an endoglucanase, endoIV, with xyloglucanase activity was identified (Vincken *et al*. 1997), but no significant sequence similarity was detected between this enzyme and EglC.

The synergistic action of endoIV and cellobiohydrolase in *T. viride* solubilised twice as much cellobiose compared to a combination of endoI, which degrades carboxymethyl cellulose and crystalline cellulose, and cellobiohydrolase. Apparently, the removal of xyloglucan from the cellulose microfibrils by endoIV is essential for an efficient degradation of cellulose in a complex matrix. (Vincken *et al*., 1994). Two other endoglucanases from *A. niger*, EglA and EglB, are controlled by XlnR (van Peij *et al*. 1998b). Both enzymes exhibit highest activity towards β-glucan but no activity towards xyloglucan. On the other hand, EglC showed only low enzyme activity towards β-glucan. It is therefore in the line of expectation that no synergy in enzyme activity of EglA, EglB and EglC was found towards β-glucan or xyloglucan used as sole substrate (Table 2, Chapter 3). However, for an optimal degradation of the complete polysaccharide-matrix in plant cell wall, a synergistic action of the different endoglucanases and

cellobiohydrolases, as was shown for endoI, endoIV, and Cbh from *T. viride*, is desired. To accomplish this co-ordinate degradation, the transcription of the genes encoding these enzymes is controlled by XlnR.

EstA, a new carboxylesterase

Several fragments of a gene encoding an unknown protein were isolated from the differential library of XlnR-controlled cDNA fragments. The complete gene was isolated and sequenced and its deduced amino acid sequence was determined to consist of 538 residues. The protein was named EstA since it is related to the α/β-hydrolase fold family of proteins, which also includes many lipases and esterases with various substrate specificities (Cousin *et al*., 1997, Holmquist, 2000). These esterases and lipases, including fungal lipases and cholinesterases, act on carboxylic esters. Their catalytic apparatus involves three residues (catalytic triad): a serine, a glutamate or aspartate and a histidine, which are responsible for the nucleophilic attack on the carbonyl carbon atom of the ester bond. EstA also contains a lipase-like catalytic triad, Ser210, Glu338 and His440, suggesting that the protein is a functional enzyme.

The *estA* gene was over-expressed in *A. niger* and the protein was purified. Crystals were generated and their structure was solved to a refined resolution of 2.2 Å (Pascale Marchot, Laboratoire de Biochimie, Institut Federatif de Recherche Jean Roche, Universite de la Mediterranee, Marseille, France). The EstA sequence showed a hybrid character; separate analysis of the amino- and carboxy-terminal halves of the EstA sequence, suggest that EstA could be an evolutional intermediate between the esterases and lipases. Analysis of the structure confirmed this hybrid character and resulted in a template for a predictive analysis of the activity and substrate specificity.

Based on the EstA catalytic centre geometry, a limited range of potential substrates was selected and assayed for EstA activity. Significant specific activities were found only with substrates of the vinyl ester and triacylglycerol groups in the order vinyl-acetate > vinyl-propionate > tripropionin > vinyl-butyrate, indicating that EstA prefers short chain substrates. Most of these substrates were also found to be hydrolysed by mouse acetylcholinesterase and pig liver esterase, albeit with distinct efficiencies. Hence EstA, in addition to being a structural intermediate between the esterases and lipases, is also a functional intermediate within the α/β-hydrolase fold family of proteins. Although its exact enzymatic function is still unknown, it is found that the transcription of the *estA* gene is regulated by XlnR. Therefore a role of EstA in plant cell wall (polysaccharide) degradation can be expected. It might be involved in processing

the lipids associated with the wall for strength and waterproofing, such as cutin, suberin or wax (Carvalho *et al*., 1998, Moire *et al*., 1999, Bernhards, 2002)

The importance of XlnR

XlnR from *A. niger*, which activates the expression of xylanolytic and cellulolytic genes, was the first fungal transcriptional regulator that was isolated. The commercial interest of xylanases and cellulases led to the isolation of several XlnR orthologues from other *Aspergillus* species as *A. kawachii*, *A. oryzae* and *A. nidulans* (Iwashita *et al*., 2001, Marui *et al*., 2002, Tamayo *et al*., 2003). A search in the Genome Database from *Aspergillus fumigatis* and *Neurospora crassa* also showed the presence XlnR proteins in these species. Also in the fungal pathogen *Magnaporthe grisea*, the expression of xylanolytic enzymes is regulated via a XlnR orthologue (http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/ Whitehead Institute/MIT Center for Genome research). Fungal pathogens secrete polysaccharide-degrading enzymes during infection of plant tissues to break down the primary cell wall of the host. *Magnaporthe grisea*, which causes the rice blast disease, secretes two distinct *endo*-beta-1,4-D-xylanases when grown on xylan-rich rice cell walls as the carbon source (Wu *et al*., 1995). Only recently the XlnR homologue from *Trichoderma reesei* was isolated (Rauscher *et al*., 2002). This fungus is known as an efficient producer of cellulolytic and xylanolytic enzymes. Two regulatory enzymes involved in cellulase and xylanase expression in *T. reesei* were isolated earlier, Ace2 being an activator, and Ace1 being a repressor of cellulase and xylanase genes (Saloheimo *et al*., 2000, Aro *et al*., 2001 Aro *et al*., 2003). Deletion of the *ace2* gene in a strain deleted for *ace1* did not reduce cellulase expression, which already indicates the presence of an additional cellulase and xylanase activator. This activator could very well be XlnR. The presence of XlnR homologues in the fungi described above indicates that the transcription regulator XlnR fulfils an extensive role in cellulase and xylanase expression in fungi.

Mechanisms involved in XlnR-controlled transcription regulation

Being a transcription activator, XlnR is able to activate the transcription of genes encoding enzymes that are required for the degradation of certain polysaccharides. This process of transcription activation requires the action of a complex regulatory apparatus in which many

different proteins are involved. Another requirement is that no energetically more favourable carbon sources, such as D-glucose, are present in the environment. In that case, the repressor protein CreA represses the transcription of the XlnR-controlled genes (Gielkens *et al*., 1997, de Graaff *et al*., 1994).

Regulation of XlnR activity

In XlnR-controlled regulation of transcription, CreA repression was described to act at both at the level of the structural genes and at the level of the regulatory gene *xlnR* (de Graaff *et al*., 1994). The suggestion for a second route of CreA repression *via* the *xlnR* gene was based on the finding that the *A. tubingensis xlnA* gene in *A. niger* was still repressed after deleting the URS from its promoter. However, there is no direct experimental evidence that CreA represses the transcription of the *xlnR* gene, thus repression *via* XlnR could also take place at another, *e.g.* post-transcriptional level. The analysis of a XlnR regulatory mutant provided us with evidence concerning the mechanism of regulation of XlnR activity. Sequence analysis of this mutant showed that a single amino acid substitution in XlnR, V756F, resulted in constitutive expression of the xylanolytic enzyme system, which means that there is transcription under non-inducing conditions (Chapter 5, Fig.1). When this mutation was introduced in the *xlnR* gene controlled by the constitutive *pkiA* promoter, transcript of the *xlnB* gene was found, even though *xlnB* transcription was still subjected to CCR *via* CreA. (Chapter 5, Fig 2). Under these circumstances no route-specific regulation can take place *via* transcriptional control of the *xlnR* gene, thus *xlnB* transcription must result from a post-transcriptional alteration, mimicked by mutation Val 756Phe, that transforms XlnR into an activated state.

CreA not only represses transcription in the presence of D-glucose, on D-xylose it also modifies the expression levels of the XlnR-controlled genes (de Vries *et al*., 1999). This was confirmed by the finding that in a CreA-derepressed strain, transcription of the XlnR-controlled genes increased strongly under inducing conditions, compared to the transcription levels in the wild type strain (Chapter 5, Fig. 5, panel E). The balance between XlnR induction and CreA repression, that determines the level of transcription in the wild type strain, is lost in this CreA-derepressed strain. When XlnR harbouring mutation Val756Phe was introduced in the CreA-derepressed strain, high transcript levels of XlnR-controlled genes were found, whether or not inducer, repressor or both are present in the medium (Chapter 5, Figures 5 and 6). It seems that this single amino acid mutation makes XlnR insensitive towards repressing signals and transforms XlnR into an irreversible active state (Chapter 5, Fig. 7).

Structure-function relations in XlnR

XlnR regulatory domains

How cans a single amino substitution such as Val756Phe, transform XlnR into a protein with constitutive activity? Does this mutation disable an on/off switch in XlnR that otherwise responds to repressing or activating signals? Besides mutation Val756Phe, deletion of the C-terminus from amino acid Leu668 resulted in constitutive XlnR activity (Chapter 6, Fig. 5d, e). Both mutants indicated that the C-terminal region of XlnR is involved in modulation of the XlnR activity. Sequence alignments of XlnR homologues from several fungi showed that the C-terminus from residue Ser699 onward is strongly conserved. Based on data presented in Chapters 5 and 6 we proposed the presence of a D-glucose inhibition domain in the C-terminus of XlnR. Mutation Val756Phe could be situated in or near this repression domain and affect its functionality (Chapter 6, Fig 6a).

Most repression domains are characterised by the presence of hydrophobic amino acids (Roberts, 2000). Mutation Val756Phe in XlnR is also located in a hydrophobic region. The percentage of hydrophobic amino acids in the complete protein sequence is 37 %, while the region surrounding Val756 contains 54 to 72 % hydrophobic residues (see table 2). There is evidence, *e.g.* from *Drosophila* repressors Engrailed, Krüppel, and Even-skipped, that the part of the repressor domain responsible for repression is alanine-rich (Han and Manley, 1993, Licht *et al*., 1990, Austin and Biggin, 1995). These domains also contain a high proportion of glutamine and proline (Gerber *et al*., 1994). In the close proximity of mutation Val756Phe, seven alanines can be found. However, no high number of prolines and glutamines can be found in the same region.

Table 2: Percentage of hydrophobic amino acids (Ala, Val, Ile, Leu, Met, Phe, Trp, and Cys) in the XlnR protein, which is 875 amino acids in size, and in parts of its C-terminus.

Amino acid position in XlnR	**Percentage hydrophobic amino acids**
1 - 875	**37**
705 - 823	54
748 - 794	63
748 - 766	72

Based on the assumption that XlnR harbours a D-glucose inhibition domain, XlnR could be able of both inducing and repressing its structural genes. Several transcription regulators contain both

activation and repression domains. One example is the *Drosophila* Krüppel protein, which switches from an activator to a repressor of transcription as its concentration within a cell increases (Roberts and Green, 1995). This process depends on the dimerisation of Krüppel, as a monomer it activates transcription, and as a dimer, it represses transcription. Another transcription factor that shows a dual function is Leu3 from *S. cerevisiae*; in the presence of α-isopropylmalate, it is a transcriptional activator and in its absence it acts as a repressor (Kohlhaw, 2003). Leu3 is localised in the nucleus and bound to its DNA target sites under different growth conditions, whether or not α-isopropylmalate is present in the medium (Kirkpatrick and Schimmel. 1995).

Modulation of XlnR activity

Phosphorylation/dephosphorylation

The ability to regulate transcription can be provided in several ways. Most of the mechanisms that control transcription factor activity involve protein phosphorylation and dephosphorylation (Whitmarsh and Davis, 2000). In XlnR, a tyrosine at position 864 was found to be essential for activation of XlnR, mutation Tyr864Asp resulted in a xylanase non-producing mutant and replacing Tyr864 by Phe strongly decreased xylanase activity on D-xylose (Chapter 6, Fig. 5c). Next to serines and threonines, tyrosines are known to be subjects of phosphorylation in proteins. Using NetPhos 2.0 Server (http://www.cbs.dtu.dk/cgi-bin), putative tyrosine phosphorylation sites in XlnR were predicted. According to this program, the change for Tyr864 being a phosphorylation site is quite low; it scored 0.016 in the range 0.000-1.000. Only three tyrosines in XlnR with high probability of being phosphorylation sites (above the treshold of 0.500) were predicted, Tyr415, Tyr464 and Tyr664. The latter one, which has a high score of 0.949, was replaced by a phenylalanine. However, since this amino acid substitution did not affect the functionality of XlnR (Chapter 6, Fig. 2c) Tyr664 is not expected to be a functional tyrosine phosphorylation site. In addition, 7 threonines and 40 serines were, based on the NetPhos 2.0 Server, assigned to be potential phosphorylation sites. From the 40 serines that could be subjected to phosphorylation, 16 scored above 0.975 (in the range 0.000-1.000), which indicate a very likely phosphorylation site. However, until now no phosphorylation site was identified in XlnR

Covalent attachments of proteins

Besides phosphorylation, covalent attachments of proteins can be involved in the regulatory processes of transcription factors. In most cases, lysine residues are targets of these covalent modifications, which include acetylation, methylation, ubiquitination and sumolyation. The

latter one, which is the process of conjugating SUMO, a Small Ubiquitin-related MOdifier, is often involved in subcellular localisation and stabilisation of transcription factors (Freiman and Tjian, 2003). Using the Abgent sumolyation calculator (SUMOplot prediction; http:/www.abgent.com/cgi-bin/tools.pl), two SUMO motifs with high probability were found in XlnR, L**K**LG from amino acid 482 till 485 and G**K**WD at position 728 till 731. The latter one could be located in or near the proposed hydrophobic repression domain of XlnR. A transcription factor that is regulated through sumolyation of a specific lysine residue in its inhibitory domain is Sp3, a member of the Sp1 family of GC box binding transcription regulators. Post-translational modification by SUMO-1 is the regulatory switch that determines whether or not Sp3 functions as an activator. It directly represses the Sp3 transcriptional activity by providing novel interaction sites, which target the protein to particular subnuclear domains (Ross *et al*., 2002, Sapetschnig *et al*., 2002). This mechanism of subnuclear localisation however is not likely to be responsible for suppression of XlnR-dependent activation by its inhibitory domain. Experiment with a Gfp-tagged fluorescent XlnR protein showed that both in the presence of inducer and repressor, XlnR is localised in the nucleus (Chapter 5, Fig. 3). In one of the XlnR mutants, in which the Val756Phe-mutated *xlnR*-gene was driven by the *pkiA* promoter, fluorescent dots were seen (Fig. 4). However, these dots did not seem to be located in the nucleus (unpublished results).

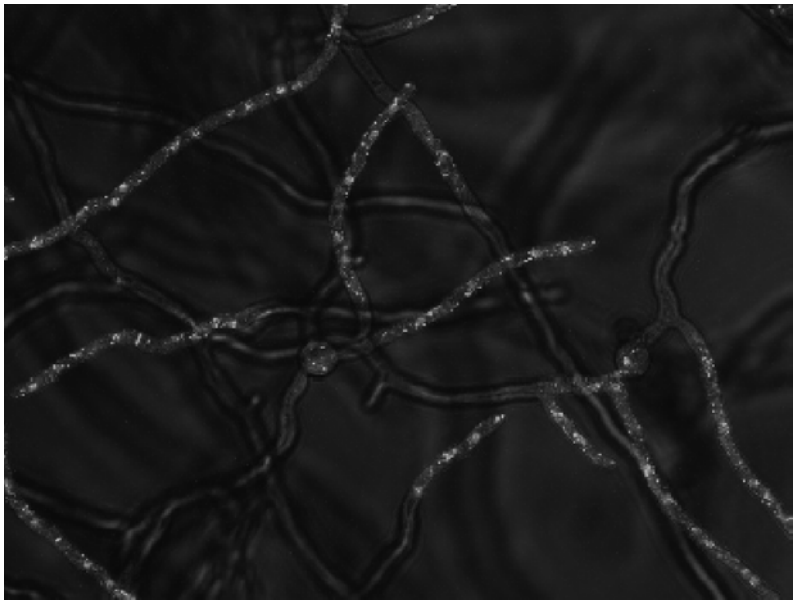

Fig. 4: Cellular localisation of Val756Phe-mutated Gfp-XlnR fusion protein driven by the *pkiA* promoter. The strain was grown in a cover glass containing MM with 10 mM D-xylose and 10 mM D-glucose for 24 h. Magnification is 40x.

Intramolecular interaction

In the activation domain of most transcription factors several acidic and hydrophobic residues are found. An example is the activation domain of the Gal4 protein, which is restricted to the C-terminal 28 amino acids; deletion of this region decreases Gal4-mediated activity with 97% (Gill and Ptashne, 1987, Swaffield *et al*., 1992). The activation domain of Leu3 encompasses only 19 residues, including three acidic and four hydrophobic amino acids (Wang *et al*., 1997). In the last 28 C-terminal amino acids from XlnR, five acidic and seven hydrophobic residues are found. However, deletion of this region did not result in decreased XlnR activity; in fact, deletion of the XlnR-terminus from amino acids Leu668, Gly797 (Chapter 6) or Phe850 (unpublished results) resulted in increased XlnR activity. This raises the question whether the C-terminus of XlnR, as was suggested before, indeed harbours an activation domain. As mentioned in Chapter 4, a proline-rich region is located from amino acids 298 till 338. As in the human CTF protein (Mermod *et al*., 1989), this proline-rich region might be located in an activation domain of XlnR.

While deletion of the XlnR C-terminus from amino acids Leu668, Gly797 or Phe850 resulted in increased XlnR activity, deletion of an internal fragment, amino acids 802 till 836, completely abolished XlnR activity. These conflicting results can be explained by a mechanism in which intra- (or inter-) molecular interactions in the C-terminus determine if XlnR is in an activated or inactivated state. In Figure 5 a hypothetical model of C-terminal intramolecular interactions in XlnR is presented. A proposed D-glucose inhibition domain in the C-terminus, downstream from amino acid Leu668, responds to the presence of repressing signals *via* intra- (or inter-) molecular interactions which turn XlnR into an inactivated state (Fig. 5a). Deletion of parts of the C-terminus disables this mechanism and results in an irreversibly activated XlnR (Fig. 5b). With the same effect, mutation Val756Phe could disable this inhibitory response mechanism. Deletion of an intermediate part of the C-terminus could, on the other hand, disturb the secondary structure of the protein in such a way that for example, as a result, the interaction of XlnR with the transcription machinery is obstructed (Fig. 5c). Otherwise disturbance of the secondary structure might result in an unstable protein.

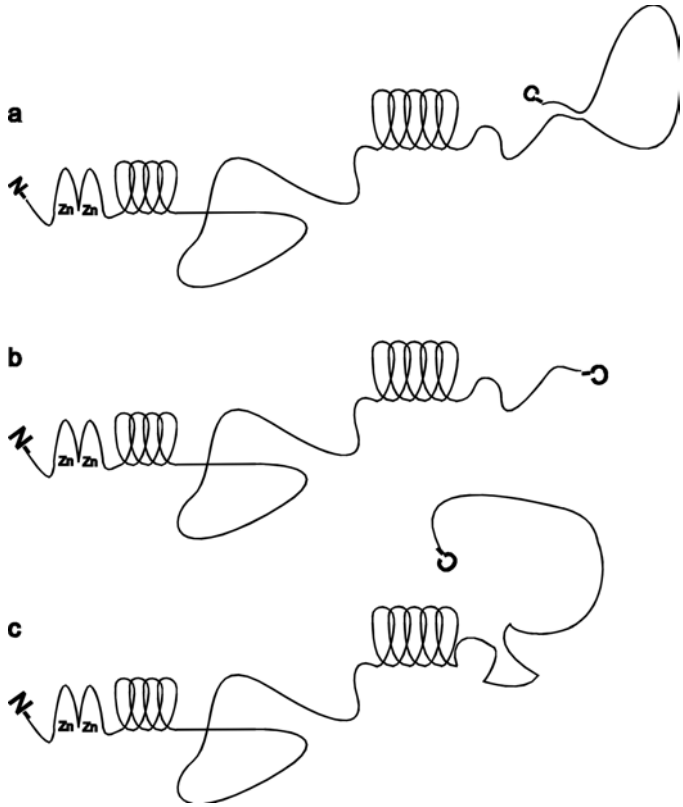

Fig. 5. Hypothetical model of XlnR. a. XlnR activity is repressed as a result of intramolecular interactions in the C-terminus. b. Deletion of the last C-terminal residues will prevent these intramolecular interactions but maintains the secondary structure of the protein. c. Deletion of an intermediate part of the C-terminus will disturb the secondary structure and might, as a result, disturb the interaction with the transcription machinery.

Nuclear import of XlnR

A number of transcription factors shuttle between the cytoplasm and the nucleus and that way regulate the expression of their target genes, such as Pho4 from *S. cerevisiae* (Komeili and O'shea, 1999) and Cre1 from *Sclerotinia sclerotiorum* (Vautard-Mey *et al*., 1999). This process of nucleocytoplasmatic shuttling relies on the recognition of nuclear localisation signals (NLSs) and nuclear export signals within the transcription factor by proteins of the nuclear import and export machinery. Phosphorylation and dephosphorylation often regulate the accessibility of the internal signals to nuclear import and export proteins (Cartwright and Helin, 2000, Whitmarsh and Davis, 2000). In Chapter 5 (Fig. 3) it was shown that XlnR is constitutively present in the nucleus and thus not regulated by nucleoplasmatic shuttling. Nevertheless, XlnR needs a functional NLS for its transport to the nucleus. *Via* deletion analysis, a coiled-coil region in the C-terminus of XlnR was determined to be involved in the process of nuclear import. After deletion of this coiled-coil domain, XlnR is retained in the cytoplasm (Chapter 6, Fig 3). Although the most frequently encountered NLS is made up of short stretches of basic amino

acids, as in PrnA from *Aspergillus nidulans* (Pokorska *et al*., 2000), in XlnR this seems not to be the case. Still, although we now know that a predicted coiled-coil domain is involved in nuclear import, the exact position of the NLS(s) in XlnR needs to be determined.

In conclusion

In the research done in this thesis, the selection of genes controlled by XlnR is expanded from eleven to fifteen genes. Two of these genes encode enzymes of which the exact function remains to be determined. Based on sequence similarities, these enzymes belong to specific classes of enzymes; one that hydrolyses ester bonds and another one that catalyses aldol condensation reactions. Considering the diverse composition of polysaccharides in plant cell walls, other genes encoding enzymes involved in plant cell wall degradation could be regulated by XlnR. Besides that, XlnR might regulate genes which encode for enzymes that play a role in specific sugar transport across the cell membrane or that functions as co-regulators. Expression profiling (DNA micro-array analysis) and proteomics could help identifying new XlnR-regulated genes and discover which enzymes are involved in the XlnR signalling pathway. It is clear now that XlnR plays a major role in regulating of the process of cellulose and hemicellulose degradation and utilisation. It controls the expression of a wide selection of enzymes both at extra- and intracellular level and therefore provides the fungus with an efficient alternative carbon utilisation system. XlnR orthologues have now been identified in several other fungi such as *A. oryzae, N. crassa* and *T. reesei*, but also in the fungal pathogen *M. grisea*. Apparently, its role in carbon utilisation is widespread in nature.

In the beginning of this research, the *xlnR* gene was presumed to be (auto-) regulated at transcriptional level. This would result in a double lock mechanism controlling the transcription of XlnR-regulated genes, as was described for AlcA/AlcR from *A. nidulans*. However, in this thesis, strong evidence was presented that XlnR is regulated at post-translational level. A single amino acid mutation in XlnR resulted in an irreversibly activated enzyme, besides that, XlnR is localised in the nucleus, both under inducing and repressing conditions. To understand its precise mode of regulation, the post-translational modifications that are involved the regulatory process of XlnR need to be elucidated. Bioinformatic methods can predict modification sites *in silico*, however, direct experimental data are still essential. Post-translational modifications can be identified using standard molecular techniques, such as deletion of the amino acid bearing the substitution, or directly using biochemical techniques, such as mass spectrometry. Proteomic

techniques might open new possibilities for the analysis of post-translational modifications. Recently strategies were developed to characterise modified 'subproteomes', such as phosphorylated proteins, with affinity-based enrichment and mass spectrometry (Mann and Jensen, 2003). Integration of the *in vivo* modification sites defined using proteomics, into bioinformatics prediction tools, will improve the identification of post-translational modifications in the future.

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SAMENVATTING

Samenvatting

Aspergillus niger is een hyfen-vormende schimmel die zich voedt met o.a. suikers, die onder natuurlijke omstandigheden afkomstig zijn van dode plantenresten. Om deze suikers en andere organische voedingsstoffen op te kunnen nemen, scheidt hij enzymen uit die het substraat buiten de cel afbreken. *A. niger* produceert een verscheidenheid aan enzymen die commercieel interessant zijn, omdat ze gebruikt kunnen worden in industriële processen. Voorbeelden hiervan zijn pectinase, dat gebruikt wordt om fruitsappen helder te maken, xylanase om de structuur van brood te verbeteren en fytase dat het fosfaat uit de plantaardige voedingstoffen beschikbaar maakt, waardoor er minder fosfaat aan veevoer toegevoegd hoeft te worden.

Wanneer *A. niger* een voedingsbron tegenkomt, die bestaat uit een polymeer van suikers zoals xylan, heeft hij specifieke enzymen nodig die het molecuul D-xylose, waaruit het polymeer gevormd is, als enkel molecuul of in dimere vorm van de suikerketen afsplitsen. Om deze enzymen zo efficiënt mogelijk te produceren, regelt *A. niger* de productie ervan *via* de transcriptie-regulator XlnR. De D-xylose suikers dienen in deze situatie als signaalmolecuul die XlnR activeert en zo aanzet tot het overschrijven van de betreffende genen tot RNA, dat vervolgens vertaald wordt in eiwitten.

In eerste instantie leek XlnR specifiek betrokken te zijn bij de regulatie van genen die coderen voor enzymen die betrokken zijn bij de afbraak van xylan. Uit later onderzoek kwam naar voren dat XlnR ook de expressie regelt van genen die coderen voor enzymen die cellulose kunnen afbreken. In dit proefschrift is aangetoond dat XlnR ook een gen reguleert dat voor een enzym codeert die in staat is om weer een ander soort suikerpolymeer af te breken, namelijk xyloglucan. Verder is gebleken dat XlnR niet alleen de expressie reguleert van genen die coderen voor enzymen die door de cel worden uitgescheiden, ook de productie van enkele intracellulaire enzymen. Een voorbeeld hiervan is D-xylose reductase, dat D-xylose omzet in xylitol, wat de eerste stap is in D-xylose katabolisme in de schimmel. Xylitol komt na een paar omzettingen in de pentose fosfaat route terecht een vervolgens in de glycolyse. Een tweede intracellulair enzym waarvan de expressie gecontroleerd wordt door XlnR is een transaldolase-achtige waarvan de functie nog niet bekend is. Transaldolases beheren samen met transketolases de stroom van metabolieten in de pentose fosfaat route door een omkeerbare reactie te katalyseren. Met de identificatie van deze nieuwe door XlnR gereguleerde genen is aangetoond dat de schimmel zijn intracellulaire processen, waarbij suiker wordt omgezet en energie wordt gegenereerd, afstemt op wat hij in het milieu aangeboden krijgt door de expressie van extracellulaire en intracellulaire enzymen gecoördineerd te reguleren.

Naast *A. niger* zijn er vele andere schimmels die polymere suikerketens in plantencelwanden kunnen benutten als voedingsbron. Voorbeelden zijn *Trichoderma reesei*, die vooral bekend is vanwege zijn productie van cellulases en *Aspergillus nidulans* die vanwege het hebben van een sexuele cyclus geschikt is voor het bestuderen eukaryote processen via kruisingen. Van de laatstgenoemde schimmel is onlangs het gen geïsoleerd dat codeert voor de transcriptie- regulator die homoloog is aan XlnR van *A. niger*. Inmiddels zijn er al 7 aan XlnR van *A. niger* homologe transcriptie regulatoren gekloneerd cq. geïdentificeerd.

XlnR is een transcriptie-regulator met een DNA-bindingsdomein dat zich aan de promoter van de te reguleren genen bindt. Dit DNA-bindingsdomein van XlnR bestaat uit een binucleair zinkcluster dat teruggevonden wordt in veel andere transcriptie-factoren. De aminozuur-volgorde van dit gedeelte van XlnR is sterk geconserveerd, in tegenstelling tot de rest van het eiwit. Op grond van overeenkomst in aminozuur-volgorde met bekende transcriptie-factoren zoals GAL4 of LEU3 van *Saccharomyces cerevisiae*, is er dan ook weinig te zeggen over structuur-functie relaties binnen XlnR.

De meeste genen die door XlnR gereguleerd worden, staan ook onder controle van een gen-expressie-onderdrukkende transcriptie regulator, genaamd CreA. Dit is nodig omdat, zodra er een energetisch meer gunstige suikerbron, zoals D-glucose, als voedingsbron aanwezig is, het hele XlnR gereguleerde expressiesysteem snel uitgezet moet worden. Gebaseerd op een gen-expressie- experiment met een door XlnR gereguleerd gen werd verondersteld dat het *xlnR*-gen ook onder controle staat van CreA en dat XlnR-activiteit dus gereguleerd wordt *via* transcriptie-regulatie. Uit onderzoek beschreven in dit proefschrift is gebleken dat dit niet het geval is. Het XlnR-eiwit blijkt zich namelijk altijd in de kern van de cel te bevinden, ook als er geen activiteit van het eiwit te verwachten is, bijvoorbeeld in afwezigheid van een signaalmolecuul of in aanwezigheid van D-glucose, een represserend signaalmolecuul. Ook blijkt de wijziging van 1 enkele aminozuur in de C-terminale helft van het eiwit tot gevolg te hebben dat XlnR in een onomkeerbare geactiveerde toestand raakt. In dit geval heeft XlnR geen signaalmolecuul nodig om geactiveerd te worden. Het mechanisme dat er normaal voor zorgt dat XlnR geïnactiveerd wordt wanneer er een energetisch voordeliger suikerbron aanwezig is, lijkt met deze enkele aminozuur-mutatie uitgeschakeld te zijn.

Om een beter inzicht te krijgen in het regulatiemechanisme van XlnR, is het nodig om de relatie tussen bepaalde structuren binnen het eiwit en de functie ervan te bepalen. Met behulp van een aantal XlnR-mutanten, waarvan een stuk van de C-terminus is verwijderd, of waarvan enkele aminozuren zijn gewijzigd, is een studie gedaan naar het effect van deze mutaties op de activiteit van xylanases. Ook is gekeken naar de localisatie van het gemuteerde XlnR-eiwit in de

cel met behulp van een fluorescent label. Uit deze studies is gebleken dat de C-terminus een regulerende rol speelt binnen XlnR. Een nog onbekend gedeelte van deze C-terminus lijkt betrokken te zijn bij de inactivatie van XlnR in aanwezigheid van D-glucose. Een gebied van ca. 30 aminozuren, dat vooraf gaat aan het C-terminale regulatie gebied en waarvan voorspeld is dat het een α-helix vormt, is betrokken bij het transport van XlnR naar de celkern. Dit is een nieuw soort transportsignaal omdat in de meeste celkerneiwitten, dit transportsignaal in de N-terminus wordt teruggevonden in de vorm van een aantal clusters van basische aminozuren. Gebaseerd op de identificatie van aminozuur-mutaties in de C-terminus van XlnR mutanten die geen xylanase-activiteit meer vertoonden, werd verondersteld dat de C-terminus, net als in veel andere transcriptieregulatoren, het activeringsdomein van het eiwit bevat. Nu is gebleken dat het weghalen van een groot gedeelte van de C-terminus geen inactivatie van XlnR tot gevolg heeft. Een enkele C-terminale deletiemutant verloor wel zijn activiteit, echter, in dit geval was een intern stuk van de C-terminus verwijderd en niet het laatste deel dat het activeringsdomein zou bevatten. Al deze bevindingen hebben geleid tot een nieuwe theorie die stelt dat intramoleculaire interacties waarbij de C-terminus betrokken is, bepalen of XlnR zich in geactiveerde, dan wel geïnactiveerde toestand bevindt. De exacte manier waarop XlnR gereguleerd is, is nog onbekend. Een onderzoek naar post-translationele modificaties binnen XlnR zou verhelderend kunnen zijn. Ook de identificatie van nieuwe genen die coderen voor eiwitten die betrokken zijn bij de regulatie-route van XlnR zou openstaande vragen kunnen beantwoorden, bijvoorbeeld naar de betrokkenheid van co-regulatoren.

CURRICULUM VITAE

Alinda Anna Hasper werd geboren op 23 april 1969 te Leiden. In 1989 begon zij met haar studie aan de Hogere Laboratorium Opleiding te Groningen met als afstudeerrichting Biotechnologie. Tijdens deze studie liep zij stage bij de vakgroep Genetica van de Rijksuniversiteit Groningen (Dr. J. Kooistra, Dr. G. Venema). In 1993 voltooide zij deze studie waarna zij haar opleiding voortzette aan de Rijksuniversiteit van Groningen. In 1996 behaalde zij het doctoraalgetuigschrift in de Biologie met als hoofdrichting Biotechnologie. De afstudeeronderzoeken werden verricht bij de vakgroep Microbiologie van de Rijksuniversiteit Groningen (Dr. J.S. Lolkema, Dr. W.N. Konings) en het Public Health Research Institute te New York (Dr. D.S. Perlin). Na het afsluiten van haar universitaire studie werkte zij tot 1997 als Associate Researcher aan het Public Health Research Institute te New York. Van 1997 tot 2002 was zij werkzaam als assistent in opleiding (AiO) bij de sectie Moleculaire Genetica van Industriële Micro-organismen, later als sectie Fungal Genomics toegevoegd aan de leerstoelgroep Microbiologie. In deze periode heeft zij onder begeleiding van Dr.Ir. L.H. de Graaff het onderzoek uitgevoerd dat beschreven staat in dit proefschrift. Sinds mei 2003 werkt zij bij Centocor BV te Leiden als Officer Quality Assurance Batch Disposition.

Nawoord

Toen ik mei 1996 klaar was met m'n universitaire studie in Groningen heb ik eerst even moeten nadenken over wat ik nu eigenlijk wilde, verder in de wetenschap of misschien wat anders. Dat nadenken heb ik een jaar lang in New York kunnen doen en heeft geleid tot de keuze om op zoek te gaan naar een leuk promotie onderzoek. Dat vond ik in Wageningen waar ik het erg naar m'n zin heb gehad en veel heb geleerd. Nu, zes jaar later, kan ik eindelijk de laatste stap nemen in het afronden van mijn promotieonderzoek, het verdedigen van mijn proefschrift.

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